THE BARN OWL (*Tyto alba*) AS A BIOMONITOR OF ENVIRONMENTAL CONTAMINATION WITH MERCURY AND ORGANOCHLORINE COMPOUNDS

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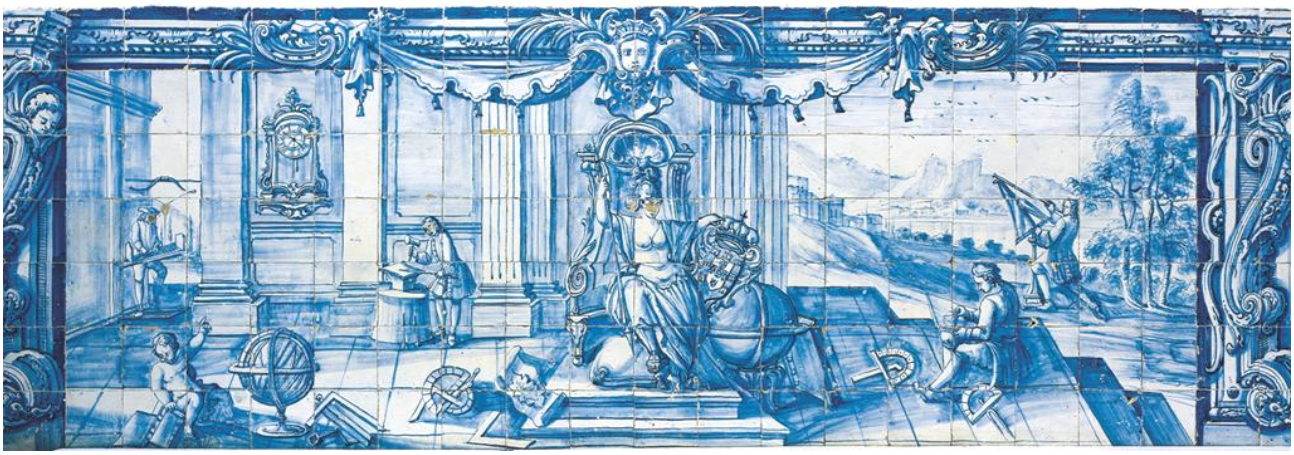

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A coruja-das-torres (*Tyto alba*) como biomonitor de contaminação ambiental com mercúrio e compostos organoclorados

Resumo

Numerosos químicos de origem industrial e natural estão a aumentar a nível global, enquanto os seus efeitos de longo-termo permanecem desconhecidos. A coruja-da-torres é um bom bioindicador de poluição ambiental e as suas penas podem ser utilizadas como ferramentas de biomonitorização minimamente invasivas. A variação intra-individual das concentrações de mercúrio pode ser minimizada através do cálculo de uma média a partir de várias penas do indivíduo, independentemente do tipo de pena. Várias penas do mesmo ninho podem ser combinadas para obter uma melhor estimativa da contaminação no território. Penas de indivíduos atropelados são representativas da contaminação ao nível regional. Menor bioacumulação está relacionada com culturas irrigadas e paisagens agrícolas heterogéneas. O habitat parece mediar a transferências de mercúrio do solo para as presas, e da cadeia trófica até à coruja-das-torres. As penas podem ser particularmente úteis para detetar pesticidas organoclorados em desuso, que geralmente ocorrem em concentrações residuais, embora a contaminação externa deva ser avaliada.

Palavras-chave: coruja-das-torres, mercúrio, pesticidas organoclorados, biomonitorização, penas

Summary

Numerous industrial and natural chemicals are increasing worldwide, while their long-term effects on wildlife and human health remain unknown. Barn owls are good biondicators of environmental pollution and their feathers may be used as minimally invasive monitoring tools. Intra-individual variation in mercury concentrations can be minimized by calculating an average from several feathers from an individual, regardless of feather type. Several feathers from the same nest may be pooled to better represent the average mercury contamination in the territory. Feathers from road-killed barn owls are representative of contamination at a regional level. Lower bioaccumulation in the barn owl is linked with irrigated crops and heterogeneous agricultural landscapes. Habitat seems to mediate the transfer of mercury from the soil to the prey, and along the food web to the barn owl. Feathers may be particularly suitable to detect legacy organochlorine pesticides which generally occur in residual concentrations, but external contamination should be assessed.

Keywords: barn owl, mercury, organochlorine pesticides, biomonitoring, feathers

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AICc	Second-order Akaike's information criterion
BAF	Bioaccumulation factors
BCFs	Bioconcentration factors
BMFs	Biomagnification factors
BSAFs	Biota-soil accumulation factors
BTFs	Biomagnification trophic factors
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
df	Degrees of freedom
EC	Emerging contaminant
EU	European Union
HCH	Hexachlorocyclohexane
Hg	Mercury
IP	Innermost primary wing feathers
IS	Innermost secondary wing feathers
MeHg	Methilmercury
OCP	Organochlorine pesticide
OP	Outermost primary wing feathers
OS	Outermost secondary wing feathers
PCBs	Polychlorinated biphenyls
P_{number}	Primary wing feather
PON	Percentage in terms of number of individuals
POP	Persistent organochlorine pollutants
SD	Standard deviation
SE	Standard error
S_{number}	Secondary wing feather

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General introduction

1 General introduction

1.1 The underlying principles of this thesis

Thousand of industrial and natural chemical compounds are increasing worldwide, while their long-term effects on wildlife and on human health remain largely unknown, which represents one of the key environmental problems facing humanity (Schwarzenbach *et al*. 2006). The increase in pesticide use as a consequence of the demands of human population growth and the ongoing increasing trend in mercury (Hg) emissions, represent a current challenge for ecotoxicological research (1.2 The ecotoxicological importance of mercury and organochlorine pesticides), because the understanding of the molecular actions of pesticides and a causal link to their possible interference with biological processes is needed, in order to develop reliable predictions about the consequences of such contaminants in a rapidly changing world (Sundseth *et al*. 2010; Köhler and Triebskorn 2013). Approximate estimates on the economic impact of contaminants has been of 8.0 billion US dollars annually for pesticides in developing countries (in non-target species, including humans; Aktar *et al*. 2009); and 8.7 billion US dollars annually in United States for methyl mercury toxicity (linked to diminished economic productivity; Trasande *et al*. 2005).

The highest concentrations of these pollutants have been found in top predators like raptors and seabirds, which respond sensitively to toxic chemicals (Becker 2003). The conspicuous decrease in raptor populations during the 1960s, in the industrialised world, alarmed ornithologists and induced research into the possible causes (1.3 Early contribution of raptors for ecotoxicology). Signs of change in bird populations (*e.g.* eggshell thinning, reproductive failure) were recognised as warning signs and were subsequently associated to pesticide use (Becker 2003). Raptors therefore performed as sentinel organisms (1.4 Ecotoxicological concepts), raising concern for environmental contamination with organochlorine pesticides (OCPs). As a consequence, the use of many of these contaminants was regulated, resulting in a decrease in their environmental levels and in a consequent recovery of the bird populations (Becker 2003; Rattner 2009; Gómez-Ramírez *et al*. 2014).

Despite the major role of ornithology in the rise of ecotoxicology (Carson 1962), when compared with other organisms, there is a disproportionate underrepresentation of birds in bioindicators literature, in opposition to a clear emphasis on plants (Burger 2006a).

Nonetheless, raptors are in general charismatic species, what reinforces their role as biomonitors: to ensure the implementation and maintenance of monitoring schemes (1.5 Raptors as biomonitors of environmental pollutants), including funding, analysis, and corrective actions, institutional compromise is essential and raptors provide sustained public and governmental interest (Burger *et al*. 1994; Burger and Gochfeld 2004; Gómez-Ramírez *et al*. 2014).

Hence, there is an urgent need of studies integrating environmental chemistry and toxicology in curricula of life sciences (Schwarzenbach *et al*. 2006). This thesis aims at contributing with information on Hg and OCPs for raptors, and more specifically in Portugal, where these studies have been rare. The overall perspective of this thesis relies on integrating the ecology of the species with matrix, individual and species specific variation in Hg and OCPs concentrations, emphasizing feathers as minimally invasive biomonitoring tools (1.6 Feathers as biomonitoring tools), in order to better understand how to control for confounding effects in opportunistic sampling procedures. Moreover, it is an attempt to understanding the importance of the barn owl (*Tyto alba*) as a biomonitor, which is associated to farmland habitats and therefore is a potential bioindicator of contamination from agricultural sources (1.7 The contribution of the barn owl).

1.2 Ecotoxicological concepts

A few ecotoxicological concepts are used in this thesis, for which it is important to have a clear definition. A bioindicator is a species or group of species that readily reflects the abiotic or biotic state of an environment, represents the impact of environmental change on a habitat, community or ecosystem or is indicative of the diversity of a subset of taxa or the whole diversity within an area (Gerhardt 2002). A reliable bioindicator needs to accomplish some requirements regarding its sensitiveness and its specificity to the correspondent impact. A good indicator (1) must display alterations in response to a stressor, but should not be so sensitive that changes arise when there is no cause, indicating biologically irrelevant variations, or simply varying arbitrarily; and (2) the alterations must be originated by a particular stressor (or series of stressors), which should be as well important to the welfare of the organism (Linthurst *et al*. 1995). Additionally, the alterations being measured should reflect impairment also to populations, communities and ecosystems (EPA 1997). A

good bioindicator should be easy to measure, which includes clear objectives, connection to the problem, straightforwardness of identification of important characteristics, and easiness of data collection and analysis (Burger 2006). In order to provide evaluation of populations, to give indication for management or species conservation, data on bioindicators must be collected repeatedly, *i.e.* under biomonitoring schemes. In this context, bioindicators are interchangeably designated as biomonitors. A particular type of bioindicators, which respond predictably to environmental disturbance or change, is named sentinel species (Gerhardt 2002). Their role as models for environmental exposures can derive from intentionally placing sentinel animals in specific environments or from observational studies of spontaneous animal disease in populations (Van Der Schalie *et al*. 1999; Reif 2011). A sentinel should (1) produce a quantifiable response (including accumulation of tissue residues) to the agent in question, (2) have a territory or home range that overlaps the monitored area; (3) be easily enumerated and captured; and (4) have sufficient population size and density to permit enumeration (National Research Council 1991).

The process by which contaminants are absorbed and undergo enrichment in organisms relative to that in environment, as a result of all uptake and loss processes (*e.g.* dietary and environmental uptake, fecal egestion, transfer to offspring, metabolic biotransformation and growth dilution) is named bioaccumulation (Arnot and Gobas 2006; Jorgensen 2010; Borgå *et al*. 2012). It consequently comprises the more specific processes of bioconcentration (*i.e.* direct portioning of chemicals between the medium and an organism) and biomagnification (*i.e.* uptake from the diet leading to higher concentrations in the organism than in its prey, resulting in increased chemical concentration with higher trophic position in the food chain; Gobas and Morrisson 2000; Jorgensen 2010).

1.3 The ecotoxicological importance of mercury and organochlorine pesticides

Mercury and organochlorine pesticides (OCPs) are ubiquitous chemicals susceptible to bioaccumulation, therefore representing a potential health risk to humans and wildlife (UNEP 2011; Bouland *et al*. 2012). Mercury and OCPs as dichlorodiphenyltrichloroethane (DDT) and cyclodiene insecticides (*e.g.* aldrin, dieldrin, endrin) are in the list of priority substances for which environmental quality standards were set in 2008 (Directive

2008/105/EC). Accordingly, they can be considered emerging contaminants (EC), *i.e.* harmful environmental agents whose identity, occurrence, hazards, and effects are not effectively recognized (Halden 2015). Despite Hg and most OCPs are legacy contaminants, their presence in trace concentrations in freshwater environments, in the nanogram (ng) or microgram per liter (μg L^{-1}), has recently been detected and is believed to adversely affect human health or the environment (Murray *et al*. 2010). The recently adopted Minamata Convention on Hg (in 2013), with the objective of protecting the human health and the environment from anthropogenic emissions and releases of Hg and its compounds validates the classification of Hg as an EC, given the existing risk of exposure (UNEP 2013). Additionally, some of the nine OCPs listed in the 2001 Stockholm Convention on Persistent Organic Pollutants, which called for a ban on the production, import, export, use, and disposal guidelines for most of these pollutants (The Secretariat of the Stockholm Convention 2010), can reveal a pattern of emergence, as is the case of DDT, which had its first peak of emergency concern in 1972 and a second in 2008 (Halden 2015). The multifactorial and dynamic emergence process is therefore not restricted to new substances, as a result of development and mass consumption of novel products, but may also be determined by the development of scientific methods and shifts in scientific paradigm (Halden 2015).

Mercury is a metal naturally present in the environment (prolific in coal and metal-rich geologic deposits) and also an introduced contaminant—its main anthropogenic sources are mining and fossil fuel combustion (Krabbenhoft and Sunderland 2013). In recent years, deposition is mostly derived from legacy anthropogenic Hg re-emitted from surface reservoirs (60% mainly from ocean and terrestrial soils), but also from primary anthropogenic emissions (27% mainly from mining and fossil fuel combustion) and from natural sources (13% *e.g.* from volcanoes; Amos *et al*. 2013). Terrestrial soils are the largest Hg reservoirs and its rising concentrations pose risks to the ecosystems because of the formation of the toxic methylmercury (MeHg) (Amos *et al*. 2013; Krabbenhoft and Sunderland 2013). Widespread poisoning of birds and mammals by organomercury seed dressings was also noted by the late 1960s (Rattner 2009). Methylmercury is harmful both to humans and wildlife, mainly due to neurological and immunological effects, and reproductive impairment (Burger and Gochfeld 1997; Scheuhammer *et al*. 2007). Methylation of the element can occur in aquatic environments, and so Hg ecotoxicological

studies have been focused mainly in aquatic organisms (Seewagen 2010). Nevertheless, toxicity thresholds have also been reported in terrestrial organisms in agricultural wetlands (Ackerman *et al*. 2010; Ackerman and Eagles-Smith 2010). Despite Hg compounds were banned as plant protection products in Europe since 1991 (Commission Directive 91/188/EEC), Hg availability appears to be increasing globally through atmospheric deposition (Windam-Meyers 2014).

The OCPs include the most prevalent synthetic pesticides that have been broadly used in agriculture in the second half of the 20th century (Barr and Needham 2002; Barr 2008). Insecticides and fungicides were in common use in the 1950s, and wildlife mortality was noted following pesticide use in agricultural and forest habitat (*e.g.* seed dressings for cereal crops and in insect control programs; Rattner 2009). These can be classified in three groups: (1) DDT and related compounds, (2) cyclodiene insecticides (aldrin, dieldrin, endrin, heptachlor and endosulfan) and (3) isomers of hexachlorocyclohexane (HCH) (Mitra *et al*. 2011). The known impact of these substances on humans includes neurotoxic, endocrine disruptive and carcinogenic effects (Ritter *et al*. 1995; Jaga and Dharmani 2003; Wasi *et al*. 2013). Despite OCPs concentrations detected in wildlife are infrequently considered to be a direct cause of death, these are often reported as a cause of immunosuppression, hormone disruption and disorder of the nervous and reproductive systems (Denneman and Douben 1993; Furness *et al*. 1993; Martínez-López, 2005). Moreover, the accumulation of OCPs residues in body fat reserves as a result of a long term exposure to low concentrations, is also of concern: under stressful conditions (*e.g.* migration, food shortage, etc.) resulting in a rapid depletion of fat reserves, OCPs residues are released into the bloodstream and mobilized to different organs such as the brain, where they may attain toxic levels and cause acute poisoning (Friend and Franson 1999). Despite most OCPs were banned as plant protection products in Europe between 1979 and 1988 (Directive 79/117/CEE and Ordinance 660/88), these substances are still detected decades after in the physical environment (Cerejeira *et al*. 2003; Villaverde *et al*. 2008; Cardoso *et al*. 2009; Carvalho *et al*. 2009), in food products (Correia-Sá *et al*. 2013; Blasco *et al*. 2004), in wildlife (Antunes-Maderia and Maderia 1989; Antunes and Gil 2004; Mathias *et al*. 2007; Van den Steen *et al*. 2009; Guimaraes *et al*. 2010) and in humans (Ferreira *et al*. 1990; Cruz *et al*. 2003; Lino and da Silveira 2006; Lopes *et al*. 2014).

1.4 Early contribution of raptors for ecotoxicology

Raptors have had a key role in finding the impact on wildlife of environmental chemicals, with strong repercussions in their regulation (Rattner 2009; Gómez-Ramírez *et al*. 2014). First major ecotoxicological findings with raptors occurred in the 1960s, almost three decades after the discovery of the insecticidal properties of DDT and following its exponentially increased use after the World War II (Hayes 1991). DDT was first associated to eggshell thinning in peregrine falcon (*Falco peregrinus*), sparrowhawk (*Accipiter nisus*) and golden eagle (*Aquila chrysaëtos*) in Britain (Ratcliffe 1967, 1970), followed by reports of pronounced declines in raptorial and fish-eating birds related with DDT in North America (Hickey and Anderson 1968) and of reproductive effects of DDT and dieldrin in feeding trials with raptors (Porter and Wiemeyer 1969). Also in the decade of 1960, there were the first studies relating mercury contamination in feathers of peregrine falcon, white-tailed sea eagle, long-eared owl (*Asio otus*), tawny owl (*Strix aluco*), common buzzard (*Buteo buteo*) and hen harrier (*Circus cyaneus*) with alkyl-Hg compounds used for seed dressing (Berg *et al*. 1966), and the first experiments with secondary poisoning with methyl mercury in the northern goshawk (*Accipiter gentilis*; Borg *et al*. 1970). Mercury was proved to have an accelerated accumulation along the terrestrial food web and a pronounced accumulation in the muscle of goshawks, which could result in fatal secondary poisoning (Borg *et al*. 1970).

In the subsequent decades, striking population declines observed in raptors in different countries – *e.g.* the above referred species in Britain; bald eagle (*Haliaeetus leucocephalus*) and osprey (*Pandion haliaetus*) in the USA; and white-tailed sea eagle (*Haliaeetus albicilla*) in Sweden – raised awareness for the need for a regulation on the release to the environment of OCPs as DDT, dichlorodiphenyldichloroethylene (DDE), dieldrintype insecticides and polychlorinated biphenyls (PCBs) (Ratcliffe 1970; Wiemeyer *et al*. 1993; Bowerman *et al*. 1994; Krone *et al*. 2006). Moreover, mercury was associated with reproductive impairment in golden eagles from Scotland and in bald eagles from USA (Wiemayer *et al*. 1984; Furness *et al*. 1989; Newton and Galbraith 1991). Adverse effects of other metals like lead were also focus of concern regarding populations of Californian condors (*Gymnogyps californianus*) in USA and marsh harriers (*Circus aeruginosus)* in Spain (Wiemeyer *et al*. 1998; Mateo *et al*. 1999). The literature regarding exposure and effects in raptors served in some measure as the foundation for the current ban under the Stockholm

Convention on persistent organic pollutants (Rattner 2009; Gómez-Ramírez *et al*. 2014). Moreover, raptors are considered under the ecological benefits of reducing mercury pollution by the United Nations Global Mercury Partnership, which assists implementation of the Minamata Convention (United Nations Environmental Programme 2002).

1.5 Raptors as biomonitors of environmental pollutants[1]

Raptors have been used for biomonitoring metals like Hg and OCPs in terrestrial environments, because in addition to their high trophic level (*i.e.* susceptible to biomagnification), they are long-lived (*i.e.* susceptible to bioaccumulation), widespread, territorial and many are sedentary, therefore also allowing for cross-habitat comparisons and linking measured concentrations to a source area (Esselink *et al*. 1995; Jager *et al*. 1996; Becker 2003; Evers *et al*. 2005). Moreover, raptors ecology features their populations as more vulnerable than other bird groups to some changes in ecosystems (Newton 1979). Since raptors are medium/large body sized birds, they have broader vital areas, therefore existing in relatively low densities. They also include species which reproductive strategy implies the production of a reduced number of offspring in each nesting event, and present long reproductive periods (*i.e.* k-strategy; *e.g.* Croxall and Rothery 1991). Species with larger body size have slower renewal rates, higher superposition between generations and a more stable age structure. Their population changes occur delayed but indicate environmental changes more conspicuously than species with r-strategy (Becker 2003). Also, reduced variations in abundance hinder a population recovery after a decline (Newton 1979). For these reasons raptor populations are sensitive to perturbation, since the loss of a territory has a great quantitative impact. Consequently, raptors include several threatened species, what reinforces their importance for the conservation of ecosystems (Birdlife International 2015), and their presence is considered an indicator of balanced ecosystems, which features them as barometers of ecological quality (Hardey *et al*. 2006). Raptors are vulnerable to reproductive and neurologic effects from elevated contaminant concentrations (Solonen and

[1] This section was partly based in the work published as: Pereira P, Godinho C, Roque I & Rabaça JE (2015) As aves de rapina e a gestão florestal do montado. In: O montado e as aves: boas práticas para uma gestão sustentável. LabOr – Laboratório de Ornitologia/ICAAM, Universidade de Évora, Câmara Municipal de Coruche, Coruche

Lodenius 1984; Eisler 1988; Wiemeyer *et al*. 1989; Evers *et al*. 2005) and they are often under various anthropogenic stressors (Strasser and Heath 2013).

Until recently, the coverage of the biomonitoring activities with raptors in Europe was unknown: a first compilation, in order to assess the potential for EU-wide coordinated monitoring, was recently made by the European Science Foundation Research Network EURAPMON (Research and Monitoring for and with Raptors in Europe; http://www.eurapmon.net). Only four European countries run national biomonitoring programmes: Sweden (National Environment Monitoring Programme; Helander *et al*. 2008), United Kingdom (Predatory Bird Monitoring Scheme; Walker *et al*. 2008), Finland (Bird Monitoring Programme; Koskimies 1989) and Norway (Monitoring Programme for Terrestrial Ecosystems; Gjershaug *et al*. 2008). Yet, these schemes operate independently, and therefore do not reveal trends in contamination at the European scale. Nevertheless, the potential for using raptors to monitor the effectiveness of EU directives at a pan-European scale seems to exist, given an extensive competence in this field of research supported by several isolated studies conducted in other EU countries (*e.g.* Portugal, Spain, Germany, Belgium, Italy, The Netherlands (Kenntner *et al*. 2003; Van Den Brink *et al*. 2003; Palma *et al*. 2005; Jaspers *et al*. 2006; Gómez-Ramírez *et al*. 2011). Over the last 50 years, 52 contaminants have been reported in 44 European countries. In the 15 active monitoring schemes, OCPs, PCBs, and metals/metalloids were monitored. In six of these countries, fungicides, flame retardants and anticoagulant rodenticides were also relatively frequently monitored. The raptor species most commonly monitored were common buzzard, common kestrel (*Falco tinnunculus*), golden eagle, white-tailed sea eagle, peregrine falcon, tawny owl and barn owl. The most widely analysed matrices were feathers and eggs, along with internal tissues (Gómez-Ramírez *et al*. 2014).

Regarding Portugal, information on contamination in raptors is scarse (Table 1.1). First contaminant being detected in Portuguese raptors in high concentrations was DDE in eggs of black-winged kite (*Elanus caeruleus*: 7.10–24.2 ppm; Palma 1985). Most data is centred in the griffon vulture (*Gyps fulvus*: 11 metals in blood, liver and kidney), in the common buzzard (four metals in blood, liver and kidney), and in the black kite (*Milvus milvus*; three metals in blood). For a group of five other species only Hg concentrations in feathers have been reported (Table 1.1). Zinc was the metal detected in highest

concentrations in Portuguese raptors (up to 378 µg dl^{-1} in blood) followed by Pb (up to 300 µg dl^{-1} in blood). In both cases the species affected was the griffon vulture (Carneiro 2015; Carneiro *et al*. 2015). Regarding the barn owl, existing information is restricted to Hg concentrations in feathers (1.22 ± 1.11 mg kg^{-1}; Lourenço *et al*. 2011), which are close to the average levels for the sampled Portuguese raptors (1.25 mg kg^{-1}). However, the existing information was gathered in the scope of academic studies, which were projects limited in geographic area and in time, and there is not an established biomonitoring programme with raptors.

Table 1.1 Overview of the environmental contaminants monitoring with Portuguese raptors

Cont.	Matrix	Species	N	Mean	SD	Med.	Range	Unit	Reference
DDE	eggs	Black-winged kite	3	-	-	-	7.10–24.2	ppm	Palma 1985
As	blood	Common buzzard	93	1.49	1.46	1.39	nd–8.51	µg dl^{-1}	Carneiro *et al*. 2014
		Black kite	31	4.52	5.7	2.44	nd–22.6		Carneiro 2015
		Griffon vulture	2	-	-	-	nd–0.300		
	kidney	Common buzzard	36	0.041	0.026	0.036	nd–0.112	µg g^{-1} w.w.	Carneiro *et al*. 2014
	liver		56	0.029	0.039	0.022	nd–0.281		
Cd	blood	Common buzzard	93	0.201	0.567	0.102	nd–4.45	µg dl^{-1}	Carneiro *et al*. 2014
		Griffon vulture	2	7.28	6.93	-	nd–12.2		Carneiro *et al*. 2015
	kidney	Common buzzard	36	0.373	0.381	0.216	0.009–1.70	µg g^{-1} w.w.	Carneiro *et al*. 2014
	liver		56	0.089	0.097	0.050	nd–0.450		
Hg	blood	Common buzzard	93	20.9	26.7	12.6	nd–165	µg dl^{-1}	Carneiro *et al*. 2014
		Griffon vulture	6	2.31	1.25	-	nd–4.39		Carneiro *et al*. 2015
		Black kite	31	7.49	8.46	4.13	nd–31.3		Carneiro 2015
	feathers	Bonelli's eagle	21	1.94	1.54	1.31	0.253–5.42	µg g^{-1} w.w.	Figueira *et al*. 2009
			21	1.94	1.54	-	0.250–5.42		Palma *et al*. 2005
		Barn owl	13	1.22	1.11	-	0.090–3.29	mg kg^{-1} f.w.	Lourenço *et al*. 2011
		Eagle owl	61	1.29	2.54	-	0.030–12.8		
		Little owl	15	0.640	0.510	-	0.100–2.27		
		Tawny owl	3	0.480	0.440	-	0.180–0.980		
	kidney	Common buzzard	36	0.503	0.310	0.448	nd–1.4	µg g^{-1} w.w.	Carneiro *et al*. 2014
	liver		56	0.389	0.346	0.319	nd–1.48		

Table 1.1 Overview of the environmental contaminants monitoring with Portuguese raptors (continued)

Cont.	Matrix	Species	N	Mean	SD	Med.	Range	Unit	Reference
Pb	blood	Common buzzard	93	14.7	65.1	5.86	nd–631	µg dl^{-1}	Carneiro *et al*. 2014
		Griffon vulture	24	29.7	13.19	-	4.97–300		Carneiro *et al*. 2015
		Black kite	31	19.4	29.3	10.4	1.67–184		
								µg g^{-1}	
	kidney	Common buzzard	36	0.245	0.364	0.102	nd–1.39	w.w.	Carneiro *et al*. 2014
	liver		56	0.152	0.152	0.079	nd–0.949		
Mn	blood	Griffon vulture	2	6.13	3.23	-	3.84–8.41	µg dl^{-1}	Carneiro 2015
								µg g^{-1}	
	liver		3	13.7	0.757	13.4	13.2–14.6	d.w.	
	kidney		3	7.95	1.65	8.43	6.11–9.31		
Mo	blood	Griffon vulture	2	-	-	-	-	µg dl^{-1}	Carneiro 2015
								µg g^{-1}	
	liver		3	0.703	0.116	0.720	0.580-0.810	d.w.	
	kidney		3	0.693	0.181	0.720	0.500-0.860		
Zn	blood	Griffon vulture	2	366	16.9	-	354-378	µg dl^{-1}	Carneiro 2015
								µg g^{-1}	
	liver		3	169	76.5	141	110-255	d.w.	
	kidney		3	83.8	7.82	86.0	71.1-90.2		
Cu	blood	Griffon vulture	2	49.1	21.4	-	33.9-64.3	µg dl^{-1}	Carneiro 2015
								µg g^{-1}	
	liver		3	29.5	13.4	23.0	20.6-44.9	d.w.	
	kidney		3	26.0	5.82	28.2	19.4-30.3		
Co	blood	Griffon vulture	2	-	-	-	-	µg dl^{-1}	Carneiro 2015
								µg g^{-1}	
	liver		3	0.137	0.0208	0.130	0.12-0.16	d.w.	
	kidney		3	0.337	0.131	0.350	0.20-0.26		
Se	blood	Griffon vulture	2	18.0	0.0282	-	18.0-18.1	µg dl^{-1}	Carneiro 2015
								µg g^{-1}	
	liver		3	2.36	0.946	1.88	1.75-3.45	d.w.	
	kidney		3	4.16	0.595	4.18	3.55-4.74		
Ba	blood	Griffon vulture	2	1.01	0.559	-	0.610-1.40	µg dl^{-1}	Carneiro 2015
								µg g^{-1}	
	liver		3	0.127	0.142	0.100	nd-0.280	d.w.	
	kidney		3	1.20	1.33	0.580	0.290-2.73		

1.6 Feathers as biomonitoring tools

Collecting biological samples from raptors raises ethical issues and therefore feathers have been broadly used as minimally-invasive monitoring tools for several contaminants (Solonen and Lodenius 1990; Dauwe *et al*. 2003; Martínez *et al*. 2012; Gómez-Ramírez *et al*. 2014). Feathers are the key excretory pathway for contaminants like Hg and OCPs and in some cases (*e.g.* Hg) they can hold up to 90% of the body burden (Honda *et al*. 1986; Braune 1987; Lewis and Furness 1991; Agusa *et al*. 2005). Concentrations in feathers result mostly from endogenous deposition of blood-circulating contaminants. Since the transfer of blood-circulating substances in feathers is interrupted after total feather growth, contaminants are trapped and remain stable, bonded to keratin fibbers (Furness *et al*. 1986). However, the biological meaning of the observed between-feathers variation is still unclear and therefore, criteria for feather selection in sampling procedures are not well established. Some authors recommend the use of smaller body feathers (Furness *et al*. 1986; Solonen and Lodenius 1990), while others recommend the use of consistently located flight feathers (Bortolotti 2010). Because body feathers are possible to collect without causing harmful effects to the bird, they have the advantage of being collected in both live birds and dead individuals, being good candidates for a standard assessment of environmental contamination levels. However, considering that many ecotoxicological studies rely mostly on opportunistic sampling, *i.e.* with access to a limited number and/or type of tissue samples, it is important to understand how the characteristics of the available samples affect the accuracy of the results and thus the quality of the conclusions.

Concentrations of contaminants in feathers and internal tissues can be affected by several factors (see García-Fernández *et al*. 2013) and there are ambiguous evidences in literature documenting strong (Jaspers *et al*. 2007a; Eulaers *et al*. 2011; Jaspers *et al*. 2011; Rajaei *et al*. 2011) and low significant correlations (Dauwe *et al*. 2005; Jaspers *et al*. 2007b; Jaspers *et al*. 2009; Espín *et al*. 2010; Espín *et al*. 2014) between contaminant levels in feathers and internal tissues. Since feathers reflect blood concentrations at the time of feather formation, the time elapsed until sampling should be considered when interpreting concentrations, particularly in comparisons with internal tissues (Espín *et al*. 2012; García-Fernández *et al*. 2013). Additionally, concentrations in feathers may also be affected by external contamination (Espín *et al*. 2014, 2016), which for some contaminants is negligible

(*e.g.* Hg; Burger and Gochfeld 1997; Dauwe *et al*. 2003) but in other cases may be an important source of variation (*e.g.* Pb; Cardiel *et al*. 2011).

1.7 The contribution of the barn owl

The ecological requirements of the barn owl and its closeness to humans make the species a potentially a good sentinel of environmental contamination, particularly in farmland habitats. This owl is an opportunistic predator that hunts in open farmland, feeding mostly on small mammals, and in many regions using man-made structures (*e.g.* barns, sheds, old houses) as nesting sites (Bunn *et al*. 1982; Roulin 2002). Because the same nests may be continuously monitored for long time periods, these sites are good sources of samples in the context of environmental monitoring. At nest sites, minimally-invasive biological samples (feathers and blood) can be collected from nestlings and sometimes shed flight feathers (from adults' moult) are also available. Moreover, during breeding season adults can also be captured in or near nest sites to collect this type of samples. Another straightforward source of barn owl samples for ecotoxicological studies is carcasses collected on roadsides. Owls are frequent victims of collision with vehicles, with road-killing estimates of 0.35–0.49 owls/km/year for Southern Portugal (Silva *et al*. 2008; Gomes *et al*. 2009; Grilo *et al*. 2014). Barn owls in particular are very susceptible to road-kill mortality, and near favourable hunting habitats may suffer traffic collisions in much higher proportion than other owl species (*e.g.* 50:1 long-eared owl, 38:1 little owl and 11:1 tawny owl; I. Roque, A. Marques, R. Lourenço, J.E. Rabaça unpublished data). This makes the barn owl a particularly prolific source of samples. Since both feathers (including wing feathers) and internal tissues may be collected from carcasses, the evaluationof intra-individual variation in contaminant concentrations may also be facilitated in the species.

1.8 Aims

This thesis results from a multidisciplinary collaboration between people working on ecology and conservation, environmental chemistry and veterinary sciences. The broad aim of this work is contributing with information on Hg and OCPs contamination in raptors, following two main lines:

A) Examine sources of variation in sampling procedures regarding raptor ecology, in order to better distinguish meaningful from redundant variation, and to propose solutions to minimize confounding effects on Hg concentrations in feathers.

A1) Verify if feathers of different types and also flight feathers varying in size and position in the wing show considerable variation in Hg concentrations, independently of feather age, in order to determine the best criteria for feather collection for biomonitoring Hg with the barn owl.

A2) Evaluate within-brood and within-territory age-associated variations in Hg concentrations, as well as those related with mixing samples with origin in nests and road--killed raptors, and assess representativeness of samples from road-killed barn and eagle owls at the regional level, in order to determine the best criteria to minimize possible confounding effects of feather age in Hg biomonitoring with the barn owl and the eagle owl.

B) Evaluate the suitability of the barn owl as a biomonitor of Hg and OCPs, given its association to farmland habitats and therefore its potential as bioindicator of contamination from agricultural sources.

B1) Characterize Hg bioaccumulation in a terrestrial ecosystem by comparing Hg concentrations in barn owl feathers with those in soil, in order to identify spatial patterns, assess the effect of diet composition, and assess the effect of possible habitat-related and industrial Hg sources in barn owl bioaccumulation.

B2) Evaluate the use of feathers as a non-destructive biomonitoring tool comparing OCPs concentrations with those measured in livers, and checking relative abundance and temporal trends in OCPs concentrations in two distinct regions in Portugal with differences in agricultural land uses.

2

Barn owl feathers as biomonitors of mercury: sources of variation in sampling procedures

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2 Barn owl feathers as biomonitors of mercury: sources of variation in sampling procedures

2.1 Abstract

Given their central role in mercury (Hg) excretion and suitability as reservoirs, bird feathers are useful Hg biomonitors. Nevertheless, the interpretation of Hg concentrations is still questioned as a result of a poor knowledge of feather physiology and mechanisms affecting Hg deposition. Given the constraints of feather availability to ecotoxicological studies, we tested the effect of intra-individual differences in Hg concentrations according to feather type (body *versus* flight feathers), position in the wing and size (mass and length) in order to understand how these factors could affect Hg estimates. We measured Hg concentration of 154 feathers from 28 un-moulted barn owls (*Tyto alba*), collected dead on roadsides. Median Hg concentration was 0.45 (0.076–4.5) mg kg^{-1} in body feathers, 0.44 (0.040–4.9) mg kg^{-1} in primary and 0.60 (0.042–4.7) mg kg^{-1} in secondary feathers, and we found a poor effect of feather type on intra-individual Hg levels. We also found a negative effect of wing feather mass on Hg concentration but not of feather length and of its position in the wing. We hypothesize that differences in feather growth rate may be the main driver of between-feather differences in Hg concentrations, which can have implications in the interpretation of Hg concentrations in feathers. Finally, we recommend that, whenever possible, several feathers from the same individual should be analysed. The five innermost primaries have lowest mean deviations to both between-feather and intra-individual mean Hg concentration and thus should be selected under restrictive sampling scenarios.

2.2 Introduction

Mercury (Hg) is a metal naturally present in the environment (prolific in coal and metal-rich geologic deposits) and also an introduced contaminant – its main anthropogenic sources are mining and fossil fuel combustion (Krabbenhoft and Sunderland 2013). Hg is mostly available to living organisms after conversion in its toxic organic form of methylmercury (MeHg), which is reported to be harmful both to humans and wildlife, mainly due to neurological and immunological effects, and reproductive impairment (Evans *et al*. 1982; Burger and Gochfeld 1997; Scheuhammer *et al*. 2007). Methylation of the element can

occur in aquatic environments, and so Hg ecotoxicological studies have been focused mainly in aquatic organisms (Seewagen 2010). Nevertheless, toxicity thresholds have also been reported in terrestrial organisms in agricultural wetlands (Ackerman and Eagles-Smith 2010; Ackerman *et al*. 2010). Despite Hg compounds were banned as plant protection products in Europe since 1991 (Commission Directive 91/188/EEC), Hg availability appears to be increasing globally through atmospheric deposition (Windham-Myers 2014), highlighting the urgent need for Hg contamination biomonitors in farmlands, for ecological and food safety concerns.

Given the pronounced bioaccumulation and biomagnification of Hg in food chains, the highest concentrations are often attained in top predator species (Lindberg and Odsjö 1983; Dietz *et al*. 2000; Lourenço *et al*. 2011). Both owls (Strigiformes) and diurnal raptors (Accipitriformes, Falconiformes) have been used as sentinels of environmental contamination in Europe since the late 1950's (Gómez-Ramírez *et al*. 2014), and most monitoring schemes used feathers as a non-invasive sampling method for several contaminants, including metals. Since feathers can be collected from both live and dead individuals, they are extremely versatile as reservoirs of contaminants, allowing for monitoring direct effects in contemporary populations, as well as for studying long time trends, using for instance specimens stored in museum collections (Bustnes *et al*. 2013; Gómez-Ramírez *et al*. 2014).

Feathers are the key excretory pathway for Hg in birds because they hold from 50% to more than 90% of the body Hg burden (Honda *et al*. 1986; Braune 1987; Lewis and Furness 1991; Agusa *et al*. 2005). Mercury levels in feathers result mostly from endogenous deposition of blood-circulating Hg and are not or slightly affected by external deposition (Burger and Gochfeld 1997; Dauwe *et al*. 2003). Since the transfer of blood-circulating substances in feathers is interrupted after total feather growth, Hg is trapped and remains stable, bonded to keratin fibbers, mainly in the form of MeHg (Furness *et al*. 1986).

However, the interpretation of Hg concentrations in feathers for biomonitoring purposes is not straightforward. There is no general agreement on which factors influence Hg deposition, and the biological meaning of the observed between-feathers variation is still unclear. While some authors recommend the use of smaller body feathers for Hg

quantification (Furness *et al*. 1986; Solonen and Lodenius 1990), others state that feathers cannot be indiscriminately selected and therefore flight feathers (remiges) should be used, given they can be consistently located (Bortolotti 2010). The correlations found in many bird species between Hg concentration in primary feathers and species-specific moulting sequence (*i.e.* feathers replaced earlier have higher Hg concentrations) are generally interpreted as a cause-effect pattern linked to Hg deposition: (1) circulating Hg levels drop as this metal is retained in growing feathers (Lindberg and Odsjö 1983; Furness *et al*. 1986; Dauwe *et al*. 2003); or (2) individuals select less contaminated prey during than before the moult (Lindberg and Odsjö 1983). However, it is also hypothesized that this pattern is an artefact of variation in feather mass for elements whose incorporation is time dependent, such as Hg. Thus heavier (and often longer) feathers show a more diluted concentration since they have a wider growth period (Bortolotti 2010). Moreover, there is evidence that the decrease in Hg concentrations along with the moult sequence is not generalized to all species: a study with barn owl (*Tyto alba*) primaries did not show any relationship between the two (Dauwe *et al*. 2003).

Owing to its ecological requirements and its closeness to humans, the barn owl is potentially a good sentinel of environmental Hg contamination, particularly in farmland habitats. This owl is a generalist and opportunistic predator that hunts in open farmland, feeding mostly on small mammals, and in many regions using man-made structures (*e.g.* barns, sheds, old houses) as nesting sites (Bunn *et al*. 1982; Roulin 2002). The same nests may be continuously monitored for long time periods: at nest sites, feathers can be collected from nestlings and sometimes shed flight feathers (from adults' moult) are also available (adults can also be easily captured to take feather samples). Another straightforward source of barn owl feathers for ecotoxicological analysis is collecting carcasses on roadsides. Owls are frequent victims of collision with vehicles, as shown for example by the road-killing estimates of 0.35 to 0.49 owls/km/year for Southern Portugal (Silva *et al*. 2008; Gomes *et al*. 2009; Grilo *et al*. 2014). Literature reporting Hg levels measured in owl feathers is still modest (see review in Espin *et al*. 2014), and to the best of our knowledge only a few studies have analysed Hg in barn owl feathers (Westermark *et al*. 1975; Denneman and Douben 1993; Dauwe *et al*. 2003; Lourenço *et al*. 2011). None of these studies examined the problem of feather sampling methods.

For ethical and legal reasons sampling live birds requires the use of non-invasive methods. Body feathers from the breast are therefore frequently used, since they are easy to pluck and it is possible to collect a few without causing harmful effects to the bird. Also, since body feathers can be collected from both live and dead individuals (while not possible for blood samples) these tissues are good candidates for a standard assessment of environmental contamination levels. Therefore, considering that many ecotoxicological studies rely mostly on opportunistic sampling, *i.e.* with access to a limited number and/or type of tissue samples, it is important to understand how the characteristics of the available samples affect the accuracy of the results and thus the quality of the conclusions.

Our main goal in this study is to verify if feathers of different types and also flight feathers (remiges) varying in size and position in the wing show considerable variation in Hg levels, independently of feather age, with implications in the use of barn owl feathers as biomonitors and in sampling procedures. We focused on feathers collected from road killed un-moulted barn owls (moult starts in the 2nd calendar year; Martínez *et al*. 2002), thus restricting the analysis to feathers from the same generation, which were simultaneously developed while the birds were nestlings (*i.e.* in each individual the available Hg in blood during growth is identical for all feathers). We tackled the following issues: (1) is the variation in Hg concentration between body and flight feathers small, so that these feather types can be interchangeably used to compare contamination levels in different sites?; and (2) is the Hg concentration in flight feathers similar despite feather length, mass and position in the wing, so that remiges (primary and secondary feathers) can be indiscriminately used to assess environmental Hg contamination?

2.3 Methods

2.3.1 Study area

Samples were collected along roads in central Portugal, between Vila Franca de Xira and Évora (7°53'–8°59'W; 38°32–38°59'N). The climate in the study area is Mediterranean, with mild winters and hot dry summers, and the rain period mainly concentrated in winter. Landscape is mostly plain or undulating and is dominated by cork oak *Quercus suber* and holm oak *Quercus rotundifolia* traditional woodland systems named 'montados', with varying tree density. 'Montados' are managed for different uses (*e.g.* cork extraction,

grazing, cereals), resulting in a multifunctional landscape. Agricultural areas occupy 10 to 30% of the study area and consist mainly of irrigated annual cultures, rice fields, rainfed cereal crops, vineyards and olive groves.

2.3.2 Sampling procedures

A total of 154 feathers were plucked from 28 barn owl carcasses collected on roadsides from 2009 to 2012: 28 samples of body feathers, 62 primary feathers and 64 secondary feathers. Whenever possible, five feather samples were collected from each individual: (1) at least three body feathers from the breast, and (2) one primary feather from the outermost group (P10 to P6), (3) one primary feather from the innermost group (P5 to P1), (4) one secondary feather from the outermost group (S1 to S6) and (5) another secondary feather from the innermost group (S7 to S12), in order to represent all the wing length. Feathers were stored in transparent plastic bags until analysis. We followed the feather numbering system of Martínez *et al*. (2002). Regarding position in the wing, flight feathers were numbered from 1 to 24 from the outermost primary (P10) to the innermost secondary (S14). Feather mean mass and length were obtained by weighing and measuring all flight feathers from the right wing of two barn owls in the range of extreme wing lengths for the species (277 and 296 mm; range in Martínez *et al*. 2002: 270–300 mm). Prior to weighing, feathers were dried in an oven for 2 hours at 35 °C. Feathers were weighed on a precision scale (0.1 mg) and measured with a wing ruler (1 mm).

2.3.3 Mercury analysis

Total Hg concentration in feather samples was determined by thermal atomization followed by atomic absorption spectroscopy, using an AMA-254 spectrophotometer (LECO, Czech Republic). The accuracy of the method was within 10% (95% confidence interval), and the quality control of the results was made using reference material (TORT-2). Reproducibility was checked by performing successive measurements on the same sample, which resulted in relative standard deviations always lower than 5%. For a 100 mg sample the detection limit was 0.01 ng. Given the reduced mass of a single body feather, for analytical reasons mean Hg concentration was calculated analysing a pool of body feathers per individual. Concerning single flight feathers, Hg concentration was determined using the

mean of the measurements in successive cuts starting from the distal part of the feather. All Hg concentrations are presented in mg kg^{-1} on a fresh weight basis.

2.3.4 Statistical analysis

The data were screened to detect outliers and check distribution normality of the variables (Quinn and Keough 2002), and a logarithmic transformation was applied to the variable Hg concentration. Linear mixed-effects models (Pinheiro and Bates 2000) were used in order to evaluate the variation of the mean Hg concentration (1) between body and flight feathers (sample size = 154 feathers, from 28 individuals) and (2) according to position on the wing and mass of flight feathers (sample size = 125 feathers, from 28 individuals). We included the individual as random effect in all models, since for each individual we had several feather samples. In a first analysis, feather type (body (B), primary (P) and secondary (S)) was used as the fixed factor; and in a second analysis feather position in the wing, feather mean length and feather mean mass were used as fixed effects. Since the three variables used in the second analysis were highly correlated (Pearson $r > 0.7$), competing models with one fixed effect only were built. Information-theoretic methods were used for model inference based in AICc values – second-order Akaike's information criterion (Burnham and Anderson 2002; Burnham *et al*. 2011). This criterion measures the contribution of each candidate model to explain the variation in Hg concentration, with a lower AICc scoring a best fitting model (Burnham and Anderson 2002). For each model it was calculated the number of parameters (degrees of freedom), log-likelihood value, AICc difference (ΔAICc), Akaike weight (w_i; *i.e.* the probability of each model given the data and the models considered), and evidence ratio. The random effects model (*i.e.* a model with intercept and random effects, but without fixed effects) was included in model selection to provide inferential information (Burnham *et al*. 2011). Model diagnostic plots were used to validate model results (Pinheiro and Bates 2000). All statistical analyses were conducted using R software 3.1.1 (R Core Team 2014) with packages gplots (Warnes *et al*. 2015), MuMIn (Barton 2015) and nlme (Pinheiro *et al*. 2015).

2.4 Results

2.4.1 Inter and intra-individual mercury variation

Median Hg concentration measured in 154 feather samples from 28 un-moulted barn owls, was 0.47 mg kg^{-1} (range: 0.040–4.9 mg kg^{-1}). Corresponding mean (± standard deviation; SD) Hg concentration was 0.62 ± 0.76 mg kg^{-1}. Mean Hg per individual ranged between 0.054 and 3.7 mg kg^{-1}, with a corresponding inter-individual SD of 0.70 mg kg^{-1}. Intra-individual SD in Hg concentration (*i.e.* Hg measurements in different feathers from a same individual) ranged between 0.013 and 1.7 mg kg^{-1}, with a mean intra-individual SD of 0.21 mg kg^{-1}. These results indicate that inter-individual variation in mean Hg concentration is in general higher than intra-individual variation in Hg measurements (Fig. 2.1).

2.4.2 Effect of feather type on mercury concentration

Median Hg concentration was 0.45 mg kg^{-1} in body feathers (range: 0.076–4.5; n = 29), 0.44 mg kg^{-1} in primary feathers (range: 0.040–4.9; n = 62) and 0.60 mg kg^{-1} in secondary feathers (range: 0.042–4.7; n = 63). Corresponding mean Hg concentration was 0.72 ± 0.94 mg kg^{-1} in body feathers, 0.59 ± 0.77 mg kg^{-1} in primary feathers and 0.60 ± 0.66 mg kg^{-1} in secondary feathers. Our data supported best the random effects model (w_i = 0.95) compared to the model testing the effect of feather type on Hg concentration (w_i = 0.05; Table 2.1). The evidence ratio for the two models indicated that the empirical support for the random effects model was 2.6 times that of the model including the variable feather type. These results suggest that the feather type did not have a strong effect on Hg concentration.

2.4.3 Effect of flight feather mass, length, and position in the wing on mercury concentration

Feathers with highest and lower median Hg concentration were P5 (0.78 mg kg^{-1}; range: 0.63–0.92) and P9 (0.19 mg kg^{-1}; range: 0.097–0.59), respectively. Considering mean Hg concentrations, feathers with highest and lower values were P6 (1.4 ± 1.5 mg kg^{-1}) and P9 (0.22 ± 0.14 mg kg^{-1}), respectively. Mercury concentrations apparently followed no order from inner to outer position in the wing and did not reflect a consistent between-feather pattern, *i.e.* the difference in Hg concentration between each feather and the previous one

was not systematically positive or negative regarding position in the wing (Table 2.2, Fig. 2.2). The information-theoretic analysis of the effects of feather mass, length and position in the wing on Hg concentration, showed that our data supported best the model with feather mass (wi = 0.69; evidence ratio to second best model = 2.2; Table 2.3). However, the random effects model also received some support, with a probability (wi) of 0.31 of being the best model (ΔAICc = 1.62). The models with feather length and position in the wing were little supported by our data. These results suggest that when analysing flight feathers from the same barn owl individual, feathers with lower mass may often show higher Hg concentration, however mass does not seem to have a very strong and clear effect (Table 2.4). On the other hand, both feather length and its position in the wing have no strong linear relationship with Hg concentration in barn owls.

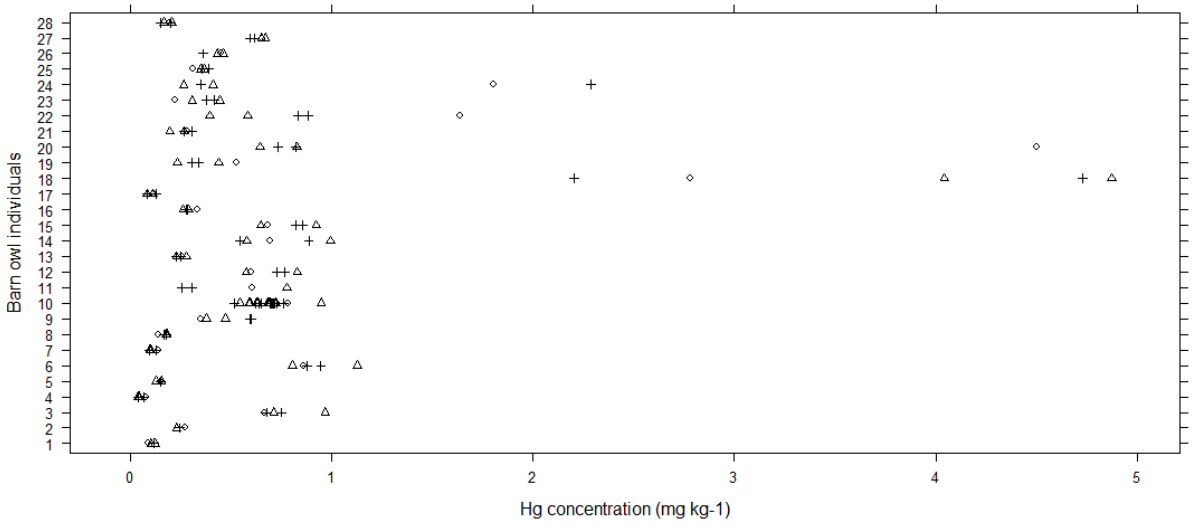

Figure 2.1 Mercury concentration (mg kg^{-1}) measurements in all barn owl individuals by feather type: body feathers (circles); primary feathers (triangles); secondary feathers (crosses)

Table 2.1 Information-theoretic model selection results for the analysis of the effect of feather type on mercury concentration in un-moulted barn owls

Model	df	log-likelihood	AICc	ΔAICc	Akaike weight (w_i)
Random effects model (intercept + random effect)	3	-90.96	188.07	0.00	0.95
Feather type + random effect	5	-91.77	193.95	5.88	0.05

Table 2.2 Mercury concentration (mean ± standad deviation, median and range), mean mass and mean length for primary (P10–P1) and secondary (S1–S13) feathers of un-moulted barn owls, ordered from the outermost to the innermost feather

Feather	Hg (mg kg^{-1})	Mean mass (g)	Mean length (mm)	Sample size
P10	0.52±0.27 0.51 (0.20–0.97)	0.4600	226	6
P9	0.24±0.18 0.19 (0.097–0.59)	0.4518	238	6
P8	0.37±0.27 0.37 (0.040–0.78)	0.4261	237	9
P7	0.44±0.22 0.38 (0.23–0.81)	0.3627	221	7
P6	1.4±1.5 0.72 (0.43–4.0)	0.3091	209	5
P5	0.78±0.21 0.78 (0.63–0.92)	0.2709	194	2
P4	0.43±0.22 0.45 (0.11–0.68)	0.2251	180	7
P3	1.1±1.6 0.56 (0.10–4.8)	0.2007	171	8
P2	0.42±0.32 0.34 (0.042–0.83)	0.1861	165	8
P1	0.45±0.30 0.48 (0.12–0.71)	0.1772	157	4
S1	0.52±0.34 0.68 (0.042–0.88)	0.1493	152	5

Table 2.2 Mercury concentration (mean ± standad deviation, median and range), mean mass and mean length for primary (P10–P1) and secondary (S1–S13) feathers of un-moulted barn owls, ordered from the outermost to the innermost feather (continued)

Feather	Hg (mg kg^{-1})	Mean mass (g)	Mean length (mm)	Sample size
S2	0.26±0.15 0.25 (0.096–0.52)	0.1542	153	7
S3	1.1±1.6 0.71 (0.13–4.7)	0.1502	153	7
S4	0.58±0.21 0.61 (0.20–0.83)	0.1396	146	6
S5	0.42±0.19 0.32 (0.26–0.62)	0.1321	144	3
S6	0.55±0.29 0.55 (0.35–0.76)	0.1222	141	2
S7	0.73±0.086 0.73 (0.65–0.82)	0.1150	137	3
S8	0.66±0.79 0.39 (0.13–2.2)	0.1118	135	6
S9	0.41±0.33 0.28 (0.067–0.89)	0.1047	137	9
S10	0.67±0.078 0.66 (0.60–0.75)	0.0979	127	4
S11	0.85±0.88 0.70 (0.082–2.3)	0.0881	124	5
S12	0.57±0.32 0.73 (0.15–0.85)	0.0733	117	5
S13	0.639	0.0493	102	1

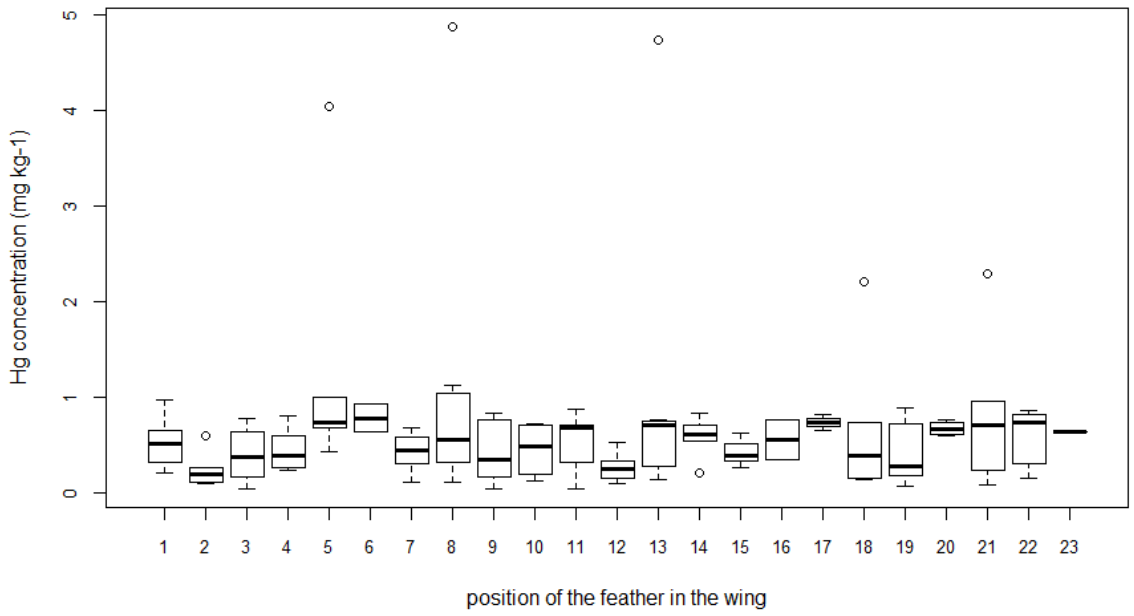

Figure 2.2 Mercury concentration (mg kg^{-1}) in barn owl feathers with different position in the wing, from outermost primary feather – P10 (1) to innermost secondary feather – S13 (23). Box and whisker plots show the median, 25% quartiles and range

Table 2.3 Information-theoretic model selection results for the analysis of the effect of feather mass, length and position in the wing on mercury concentration in un-moulted barn owls

Model	Df	log-likelihood	AICc	ΔAICc	Akaike weight (w_i)
Feather mass + random effect	4	-60.29	128.91	0.00	0.69
Random effects model (intercept + random effect)	3	-62.17	130.53	1.62	0.31
Feather position + random effect	4	-64.80	137.93	9.02	0.01
Feather length + random effect	4	-66.43	141.20	12.28	0.00

Table 2.4 Model results for the analysis on the effect of feather mass on mercury concentration in un-moulted barn owls

Fixed effect	Estimate	SE	df	t	P
Intercept	-0.92	0.17	96	-5.29	<0.001
Feather mass	-0.43	0.18	96	-2.32	0.022
Random effect	SD – intercept (between-individual variation)		SD – residuals (within-individual variation)		
Individual	0.882		0.252		

2.5 Discussion

2.5.1 Mercury contamination in barn owl feathers

In general, the Hg concentrations we measured in barn owl feathers (0.62 ± 0.76 mg kg^{-1}) were below the levels previously detected for this species in the Iberian Peninsula (1.2 ± 1.1 mg kg^{-1} in body and flight feathers, n = 13; Lourenço *et al*. 2011), in Belgium (0.77 ± 0.44 to 0.90 ± 0.53 mg kg^{-1} in primary feathers, n = 5; Dauwe 2003), in the Netherlands (1.8 ± 0.93 mg kg^{-1} in primary feather P4, n = 3; Denneman and Douben 1993) and in Sweden (15 ± 32 mg kg^{-1} in indiscriminate feathers, range 0.4–6.0 mg kg^{-1} during alkyl Hg ban and 0.19–126 mg kg^{-1} during alkyl Hg use in agriculture, n = 16; Westermark *et al*. 1975). The toxicity threshold for Hg is highly variable among bird species and reported sub-lethal effects are mainly associated with reproductive impairment (Scheuhamer *et al*. 2007). Concentrations from 2.4 mg kg^{-1} in body feathers have been reported to cause a reduction in nest success by 10% in songbirds (Jackson *et al*. 2011), whereas concentrations over 40 mg kg^{-1} are associated with sterility in the white-tailed eagle *Haliaaetus albicilla* (Berg *et al*. 1966). In our data set, 3% of samples (five samples from two individuals) showed Hg concentrations in the range of the values reported to produce negative effects on terrestrial birds (between 2.8 and 4.9 mg kg^{-1}). Therefore, despite in our study area barn owls are in general not exposed to very high Hg contamination, we should consider some of our values as sufficiently high to potentially impair reproduction. Nevertheless, we cannot completely exclude the possibility

that the highest values reported in this study could have resulted from sporadic external contamination, such as small particles retained in feathers.

2.5.2 Mercury contamination in body feathers *versus* flight feathers

Our results suggest that either body feathers from the breast, primaries and secondaries are adequate to evaluate Hg levels in first-year barn owls, since no consistent differences between these three feather types were observed. Thus, opportunistic sampling should be applicable provided mean concentrations are calculated from several feathers: given the considerable variation in Hg levels between different feathers, irrespectively of feather type, it is advisable to use more than one feather to estimate an Hg value per individual. If this procedure is adopted, it is expected that Hg concentration measured in a juvenile could be considered a reliable indicator of local contamination (*i.e.* the area surrounding the nest site).

2.5.3 Mercury concentration in flight feathers: variations with position in the wing, feather mass and length

The effect of feather position in the wing seems to be small as it showed no linear relationship with Hg variation in our data set. However, the widest range in Hg concentration was found between P9 and P5–P6, and hence we recommend caution when using the outermost primaries to estimate and compare Hg contamination in barn owls, particularly in studies with small sample sizes. Given the negative effect of feather mass on Hg concentration (due to dilution), the largest outermost primaries, being the heaviest feathers in the barn owl wing, might contain lower Hg concentrations when compared to smaller feathers. However, while feather length and mass in general decreases inwards (P10–S13; with exception of an increase in length in P10–P9 and in both mass and length in S1–S2), our results did not show a comparable trend inwards-outwards in Hg concentration in barn owl remiges, contradicting the general pattern described in the literature (see Bortolotti 2010 and references therein).

Bortolotti (2010) has demonstrated that the relationship between the position of a primary and its relative mass is the inverse of the relationship between the position of a primary and its relative Hg concentration. Based on this finding he proposed that Hg

concentration in primaries is confounded by a variation in feather mass. In his data (n = 5 individuals; adapted from Furness *et al*. 1986), Hg concentrations followed the general pattern of contaminant concentrations found in several studies: a decrease from P1 to P8 or P9 and then an increase in P10. In our study, although the relationship between the position of barn owl primaries and their relative mass and length followed a pattern similar to that found by Bortolotti (2010), the relative Hg concentrations in primaries showed a very different pattern (Fig. 2.3 a), thus supporting independence from relative feather mass and length. Despite the current poor understanding of feather physiology, Bortolotti (2010) also hypothesized that Hg passively accumulates in the feather in a time-dependent manner, *i.e.* the length of time growing cells are exposed while Hg passes from the circulation to the growing feather is critical. Hence, differences in the growth rate, *i.e.* in daily increase in feather mass and/or length, should be determinant to differences in Hg concentrations between feathers. Therefore, the pattern found by Bortolotti may not illustrate a cause--effect relationship between feather mass and Hg concentration but is eventually a consequence of the position of the primaries in his data set being correlated with the growth rates of individual feathers.

The post-moult growth of the outermost primaries was described for barn owl by Lenton (1984), and their daily increase in length and mass can be calculated from his data. The pattern of mean Hg concentration we found among the outermost primary feathers is concurrent with Hg deposition being influenced by differences in daily increase in mass and length during feather growth: both increase from P10 to P8, then decrease to P6 and rise again in P5 (Fig. 2.3 b). Mean Hg concentration showed an opposite trend, with its highest value in P6, which is the primary with the lowest daily increase in mass and length. Our data is in agreement with this rationale, since feather mass and not length showed a stronger effect on Hg concentration, and accordingly differences between feathers are more pronounced in the daily increase in mass than in length (Fig. 2.3 b). Moreover, feather mass and length do not seem to fully explain the total Hg excreted in the feather (mean Hg concentration multiplied by feather mass), since relative excreted Hg follows the pattern of Hg concentration irrespectively of feather size (Fig. 2.4). Differences in Hg concentrations can be so accentuated that a smaller and lighter feather could excrete more Hg (see for example P6 and P9). Therefore, the contribution of a single feather to Hg elimination may be more dependent on its susceptibility to incorporate Hg due to its growth rate than on its

size, suggesting that besides mass and length other factors can be determinant to the process of Hg deposition in feathers.

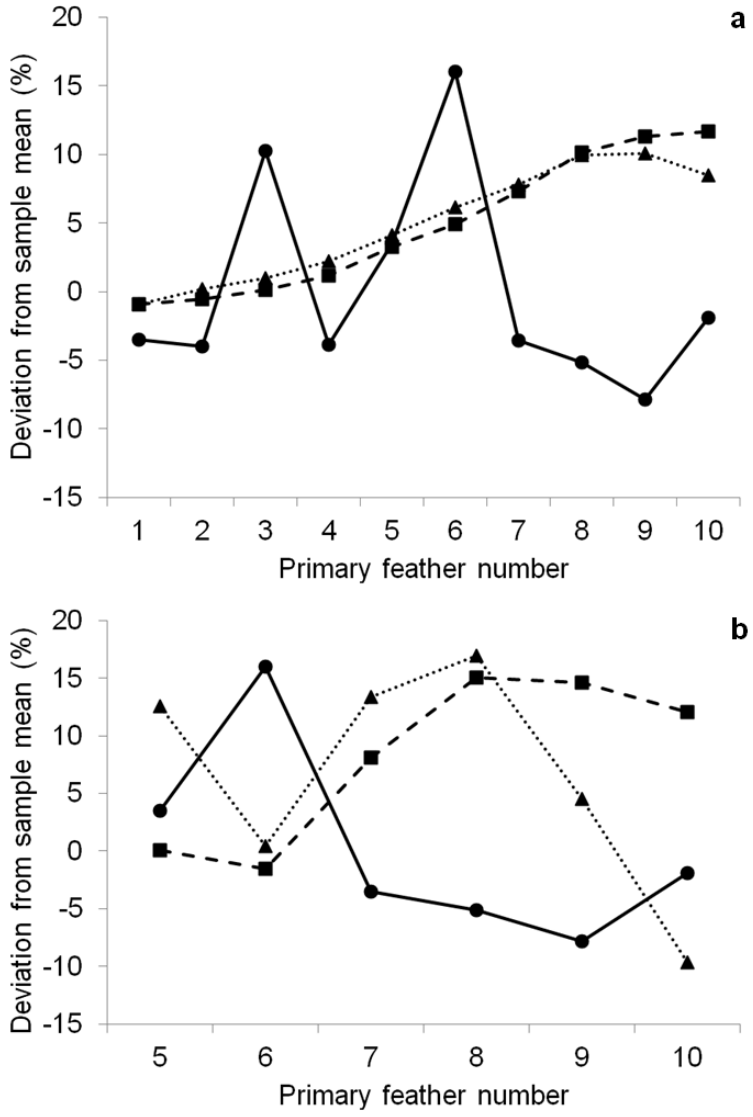

Figure 2.3 Patterns of variation in Hg concentration (circles and solid line) in barn owl primary feathers *versus* feather size (a) and growth rate (b). Squares and dashed lines represent feather mass (a) and daily increase in feather mass (b); triangles and dotted lines represent feather length (a) and daily increase in feather length (b). All values are expressed in % deviation from the sample mean. Daily increase in mass and length (b) was adapted from Lenton (1984) and was only available for the six outermost primaries (P5-P10). Lines joining points are for visual emphasis

Although in our study the feather with the lowest Hg concentration is simultaneously the longest feather in the barn owl wing (P9), in Lenton (1984) P8 was the longest feather and had the highest daily increase both in mass and length (most likely this reflects the deviation to the pattern in Fig. 3b). Since feather morphogenesis is genetically determined (Yu *et al*. 2002), we assume that feather growth pattern is equivalent in juveniles and adults. Nevertheless, further detailed studies on feather development in barn owl nestlings are needed.

Our study seems to support the hypothesis that Hg deposition is time dependent as stated by Bortolotti (2010). However, our results suggest that feather growth rate is possibly the main determinant of differences in Hg concentration found in flight feathers. Future studies with detailed data on growth rate of all flight feathers in barn owl nestlings are needed to confirm this hypothesis. As a consequence of this conclusion, the correction method suggested by Bortolotti (2010) of using length as a proxy of time for quantifying Hg in feathers (instead of concentration calculated as Hg mass divided by sample mass) may not be valid for the barn owl (and possibly for other bird species as well), since differences in feather length do not fully represent differences in feather growth rate.

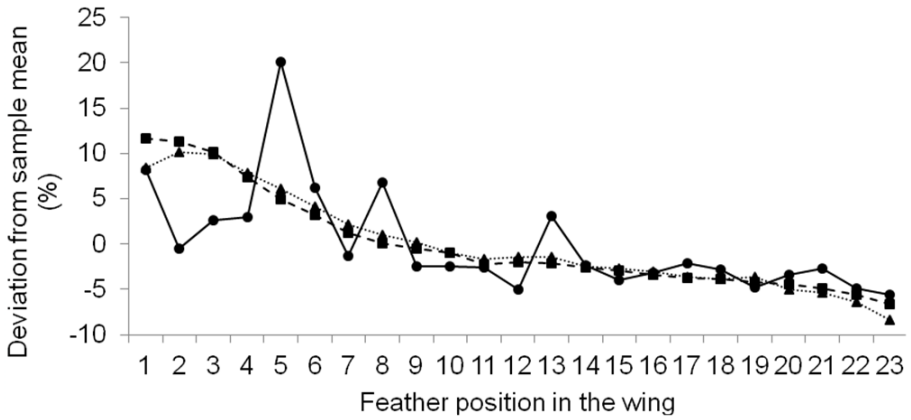

Figure 2.4 Variation in the relative estimated amount of Hg excreted (circles and solid line) in barn owl flight feathers *versus* relative feather mass (squares and dashed lines) and length (triangles and dotted lines). All values are expressed in % deviation from the sample mean. Lines joining points are for visual emphasis. Feathers are ordered from outermost primary feather – P10 (1) to innermost secondary feather – S13 (23)

2.5.4 Implications to sampling procedures

The use of barn owl feathers to biomonitor Hg contamination, as in raptors in general, is often subjected to sampling and analytical constraints. If researchers are sampling live birds, the most ethical option is to collect a few body feathers. On the other hand, in studies relying on bird carcasses or shed feathers found in nest sites and perches, the limitations are related to feather availability and the sample size that can be analysed. Such opportunistic sampling implies that for some individuals or sites only a certain type or number of feathers can be used. Sampling constraints are particularly restrictive when relying on shed feathers, because the barn owl has a complex moult and can shed a small number of feathers in some years (1–2 feathers, Martínez *et al*. 2002). Moreover, the exact position in the wing is seldom identifiable in shed feathers (exception to P10, owing to its particular structure) and also age is undetermined, meaning additional variability is introduced by possible differences in bioaccumulation when using moulted feathers.

Our results suggest that it is not crucial to discriminate between feather type, position in the wing and length, since these characteristics seem to have little importance on the feather ability to accumulate Hg. Considering that simultaneously-grown remiges (*i.e.* with equal Hg concentration available in the blood) differ in their ability to incorporate Hg because they have different growth rates, then the best estimate of individual Hg level should overcome between-feather variation. To accomplish this, a mean value should be obtained by using several feathers from the same individual. Moreover, in obtaining the best estimate possible of the individual mean Hg concentration, intra-individual variation should also be considered. Since mass has a dilution effect in Hg concentration in remiges, we grouped these feathers in four classes by decreasing mean mass, in order to examine the effect of mass in the deviations to mean Hg concentrations: the five outermost primaries (OP–0.40 g), the five innermost primaries (IP–0.21 g), the seven outermost secondaries (OS–0.14 g) and the six innermost secondaries (IS–0.088 g). The group that contributes to minimise both inter-feather and intra-individual variations includes the five innermost primaries (Fig. 2.5). Accordingly, under a restrictive scenario, *i.e.* when choices must be done on which feathers to analyse, feathers from this group seem the best possible option (*i.e.* primaries 5 to 1). Its average deviations from sample and individual mean Hg concentration are low (respectively -0.019 and -0.009 mg kg^{-1}). This roughly corresponds to remiges in the

range of length of 157 to 194 cm. Although this criterion is by principle applicable to adult birds, the increasing trend in Hg with age and the complex stepwise moult of the barn owl are probably more relevant to explain differences in Hg concentrations between feathers in adults than feather growth rate.

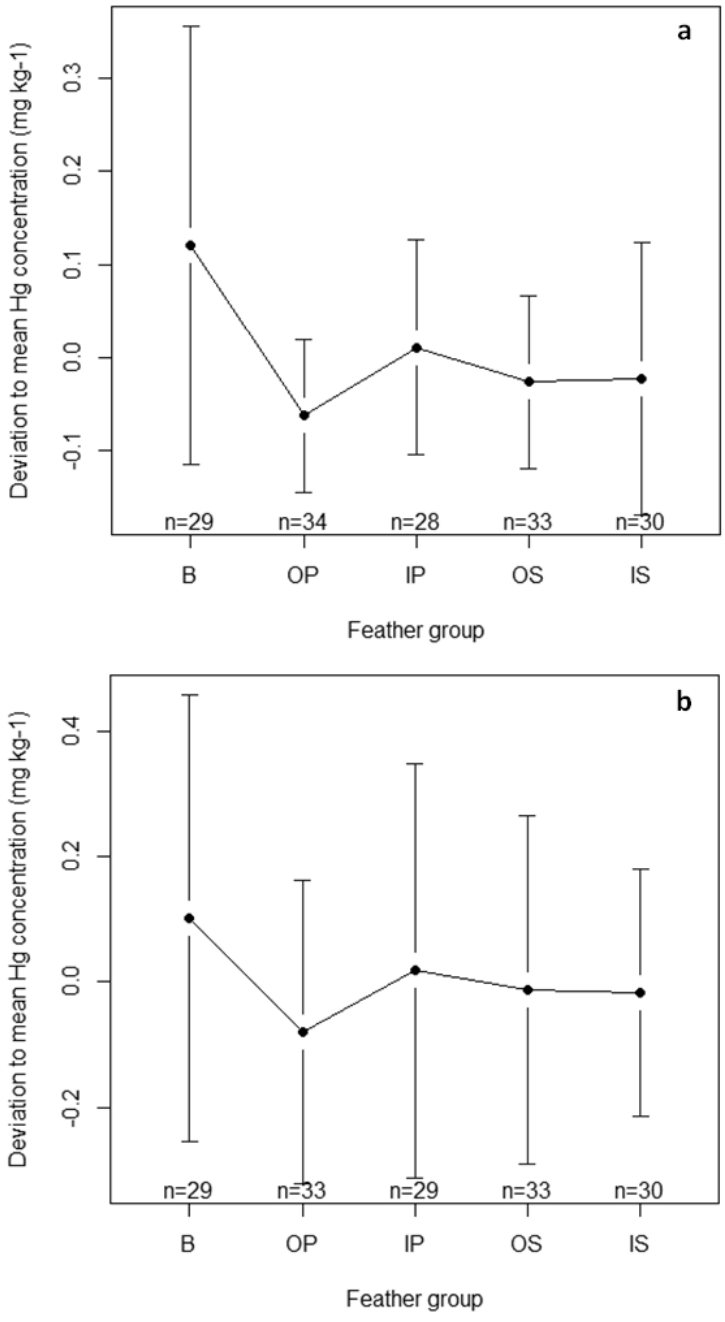

Figure 2.5 Inter-feather (a) and intra-individual (b) deviations to mean Hg concentration. B – body feathers. Remiges are grouped in mass classes: OP – mean mass 0.402 g (5 outermost primaries); IP – mean mass 0.212 g (5 innermost primaries); OS – mean mass 0.138 g (7 outermost secondaries); IS – mean mass 0.088 g (6 innermost secondaries). Dots represent mean and error lines represent 95% confidence intervals. The line adjoining dots is for visual emphasis

3

Biomonitoring mercury with owl feathers from nests and road-kills: age effects at local and regional levels

This chapter was based on the manuscript in preparation:

Roque I, Lourenço R, Marques A, Coelho JP, Cruz N, Pereira E, Roulin R, Rabaça JE. Biomonitoring mercury with owl feathers from nests and road kills: age effects at local and regional levels

3 Biomonitoring mercury with owl feathers from nests and road-kills: age effects at local and regional levels

3.1 Abstract

Raptor feathers have been widely used as non-invasive monitoring tools for several contaminants in terrestrial environments – including Hg – in part because raptor ecology allows the linkage of measured concentrations to a source area. Feather samples are mainly available from nests and road-killed birds, but these two sources may imply age-related confounding effects on Hg concentrations. To analyse age variations in Hg concentrations at local and regional levels, we used 112 feather samples from nest sites (57 barn owl feathers from 25 nests, 32 eagle owl feathers from 29 nests), and 156 feathers from 37 barn owls carcasses) collected in South Portugal between 2004 and 2012. At the local (nest-site) level, Hg concentrations were on average significantly higher in body feathers from the older barn owl sibling (0.555 ± 0.594 ng g^{-1}) than from their siblings (0.263 ± 0.189 ng g^{-1}) that were on average 7.46 ± 7.47 days younger. There were no significant differences between age classes (nestlings and adults) in barn owls and eagle owls. Our results suggest that feathers of different nestlings from the same brood, or nestlings and adult moulted feathers from the same territory may provide similar indication of Hg contamination. None of the comparisons between barn owl feathers from nests and road-killed individuals with regard to age showed significant differences. To reduce possible body mass-effects on Hg concentrations measured at the territory level (particularly in nestlings), an average should be calculated from more than one individual per nest, regardless of age class. Feathers from road-killed barn owls can apparently be collected irrespectively of age, since Hg concentrations are comparable to those measured in feathers collected in owl territories in the same area. The similarity of Hg levels between age classes may result from our study species being resident but also from most samples corresponding to feathers formed in the nest, showing no bioaccumulation effect despite differences in individuals' age. Because of the moult strategy and higher mortality of juveniles in both owl species, there is a greater likelihood of obtaining first year feathers in an opportunistic sampling scenario. Therefore, the higher variability in Hg concentrations in barn owl and eagle owl feathers from nests, and road-killed birds may likely represent local environmental contamination.

3.2 Introduction

Metals and pesticides are among the major pollutants for which a warning of increasing environmental concentrations is important (Esselink *et al*. 1995). Metal pollution is often derived from anthropogenic activities, especially in terrestrial environments (Castro *et al*. 2011). Mercury is a widespread metal apparently increasing worldwide through atmospheric deposition (Windham-Myers 2014). In recent years, deposition is mostly derived from legacy anthropogenic Hg re-emitted from surface reservoirs (60% mainly from ocean and terrestrial soils), but also from primary anthropogenic emissions (27% mainly from mining and fossil fuel combustion) and from natural sources (13% *e.g.* from volcanoes; Amos *et al*. 2013).

Raptors have been used for biomonitoring metals like Hg in terrestrial environments, because in addition to their high trophic level (*i.e.* susceptible to biomagnification), they are long-lived (*i.e.* susceptible to bioaccumulation), widespread, territorial and many species are sedentary, thus also allowing for cross-habitat comparisons and linking measured concentrations to a source area (Esselink *et al*. 1995; Jager *et al*. 1996; Becker 2003; Evers *et al*. 2005). Raptors are vulnerable to reproductive and neurologic effects from elevated Hg concentrations (Solonen and Lodenius 1984; Eisler 1988; Wiemeyer *et al*. 1989; Evers *et al*. 2005) and they are often under several anthropogenic stressors (Strasser and Heath 2013). Many raptor species have an unfavourable conservation status (Birdlife International 2015), and thus ethical issues are decisive regarding the selection of sampling methods. Consequently, raptor feathers have been broadly used as non-invasive monitoring tools for several contaminants (Solonen and Lodenius 1990; Dauwe *et al*. 2003; Martínez *et al*. 2012; Gómez-Ramírez *et al*. 2014).

Mercury is excreted in feathers and its concentrations reflect endogenous deposition of the blood-circulating metal at time of feather formation (Burger and Gochfeld 1997; Dauwe *et al*. 2003). After total feather growth, the transport of substances from blood to keratine fibers is blocked and Hg remains stable in the feather, mostly in the form of methylmercury (MeHg; Furness *et al*. 1986). Mercury concentrations in feathers are much higher than those measured in eggs, blood or muscle, despite they may be higher or lower than liver storage concentrations (DesGranges *et al*. 1998; Evers *et al*. 2005). Feathers are

considered valuable biomonitors of Hg contamination because (1) they are sampled non--destructively, (2) have chemical and physical stability, and (3) accumulate higher Hg levels than other tissues.

Apart from museum collections and birds that enter rescue centres (on which basic information is generally uncontrolled by researchers), feather samples for ecotoxicological studies may be collected at nests (either plucked from nestlings and adults or found shed) or plucked from road-killed birds. In resident species, feather samples collected at nests are allocated to a specific site, where birds have nested and fed, and can therefore reflect local contamination. On the other hand, the original nest site/territory of samples collected from road-killed individuals is not certain, although it can often coincide with the collection site. Collection site should theoretically differ more from feather-growth site in the case of first year individuals, which are most frequently found dead on road sides, mainly during post--natal dispersal (Massemin *et al*. 1998; Borda-de-Água *et al*. 2014). However, regardless of age, the bird may also be a floater, *i.e.* a non-territorial individual (Penteriani 2006; Delgado and Penteriani 2008), in which cases the collection site may also be quite far from feather--growth site. Moreover, samples from road-killed birds are often collected from degraded carcasses, where little information on the individual may be available, including age. As a consequence, in resident species, samples from road-killed birds may only be securely related to contamination at a broader regional level.

Inherently, age influences sample availability, because nestlings are easier to capture and handle, while sampling adults may depend on the access to shed feathers. Also, first year owls are more likely to die on road sides than adults (de Bruijn 1994; Massemin *et al*. 1998). Samples are often considered homogeneous to facilitate the analysis, with a consequent risk of error in the results (Castro *et al*. 2011). Metal concentrations can vary in samples from individuals of different age, which in turn may influence the validity of results by creating discrepant outcomes on the effect of age in Hg contamination. Many studies report higher Hg concentrations in feathers from adults than nestlings or sub-adults (Furness 1990; Furness *et al*. 1991; Stewart *et al*. 1994; Monteiro *et al*. 1995; Wenzel and Gabrielsen 1995; Gochfeld *et al*. 1996). However, other studies support similar concentrations between nestlings and adults (Burger *et al*. 1994; Monteiro and Furness 1995) or higher concentration in nestlings than adults (Monteiro and Furness 1995). Bioaccumulation in internal tissues can

change with age, leading to different Hg levels in blood circulation at the time of feather growth (Burger 1993). This may be responsible for age-related differences in Hg levels between feathers of juvenile and adult birds. Since a continuous sequestration in internal tissues is presumed, part of this accumulation could be mobilized into blood circulation, increasing concentrations derived from recent food (Burger 1993), and possibly resulting in adults having higher Hg concentrations in feathers. On the other hand, associations between age classes in body weight and prey-type selection (*e.g.* smaller younger juveniles foraging on smaller prey than older juveniles and/or adults) may also contribute to age-related differences in Hg exposure (Evers *et al*. 2005).

We used two resident owl species as case studies to analyse age-effects in Hg concentrations at local and regional levels. The barn owl (*Tyto alba*) is a medium-sized opportunistic meso-predator that hunts in open farmlands (Bunn *et al*. 1982; Roulin 2002). The eagle owl (*Bubo bubo*) is a large-sized generalist super-predator associated to undisturbed and often steep and scrubby areas (Cramp 1985). Since these species have distinct habitat requirements and have a different trophic position in the food web, they represent ecological variations within the owl group.

We explored within-brood and within-territory variations in Hg concentrations associated with age, as well as those related to sample-type and representativeness of road--killed samples. Accordingly, we posed four sampling-related questions: (1) Are Hg concentrations similar among siblings, so that feathers from nestlings of the same brood can be indiscriminately collected to measure Hg concentrations at the territory level? (2) Are Hg concentrations similar among age classes in related individuals, so that feathers from nestlings and adults can be interchangeably used to characterize Hg levels in a territory? (3) Are Hg concentrations similar among age classes in unrelated individuals, so that feathers from different proveniences (nests and road-killed individuals) can be indiscriminately used to measure Hg concentration in a certain region? (4) Are Hg concentrations similar between road-killed and nest barn owls with different ages, so that feathers from road-killed individuals can be used to represent a certain region, irrespectively of age of sampled individuals?

3.3 Materials and methods

The analysed feathers were collected at nest sites (either plucked from the bodies of live barn owls and eagle owls or found shed), and from dead barn owls collected on road sides in Ribatejo and Alentejo regions (South Portugal), between 2004 and 2012. A total of 112 samples were obtained in nests: 57 barn owl feathers (collected at 25 nest sites; 43 feathers from nestlings and 14 feathers from adults) and 32 eagle owl feathers (collected at 29 nest sites; 13 feathers from nestlings and 19 feathers from adults). Road killed barn owls were frozen stored at -20 °C until processing and age was determined by plumage moult according with Martínez *et al*. (2002): 156 feather samples were collected from 37 carcasses (96 feathers from 20 first year owls and 60 feathers from 17 adults). All feathers were stored in transparent plastic bags until chemical analysis.

Total Hg concentrations were measured by thermal atomization atomic absorption spectroscopy, following the same method as described in Roque *et al*. (2016). The quality control of the results was assured using reference material (TORT-2), which delivered an accuracy of 10% with 95% confidence intervals. Reproducibility was inspected by succeeding measurements on the same sample, which delivered standard deviations lower than 5%; and detection limit was 0.01 ng for a 100 mg sample. Hg concentrations are presented in ng g^{-1} on a fresh weight basis.

For Hg contamination assessment at local level, data were grouped by nest site. Non--parametric statistical tests were used because data showed a non-normal distribution that could not be normalized by transformation. Paired Wilcoxon tests were used to compare Hg concentrations in older *versus* younger barn owl siblings (*i.e.* within-brood variations) and in parents *versus* offspring in barn owls and eagle owls (*i.e.* within-territory variations).

For Hg contamination assessment at regional level, mean Hg concentration in barn owl feathers from carcasses was calculated using either four or five flight or body feathers per individual, in order to minimize intra-individual variation driven by differences in feather mass (Roque *et al*. 2016). Data normality was achieved by log-transformation. Data were also inspected for homogeneity of variances. In order to determine if different sample types (age-associated) could be indiscriminately collected to measure Hg concentration in a certain region ANOVA tests were used to compare nestlings *versus* road-killed sub-adults (1 year)

versus adults (2 or more years) in barn owls, and nestlings *versus* unrelated breeding adults in eagle owls. In order to determine if Hg concentrations in feathers from road-killed individuals are comparable with those from nests in the same region, irrespectively of the age of sampled feathers, Student's t-tests were used to compare barn owl feathers from nests (nestlings and nestlings plus adults) with road-killed individuals with one year and two or more years. All statistical analyses were conducted using R software 3.1.1 (R Core Team 2014).

3.4 Results

3.4.1 Age effect in Hg concentrations at the territory level in the barn owl and eagle owl

Within the same brood, mean difference in age of the older and younger barn owl nestling was 7.46 ± 7.47 days (range: 1–26 days) and mean difference in Hg concentration in body feathers was 0.364 ± 0.524 ng g^{-1} (range: 0.007–1.96 ng g^{-1}). Overall, Hg concentrations were significantly higher in body feathers from the older nestling (0.555 ± 0.594 ng g^{-1}) than from the younger nestling (0.263 ± 0.189 ng g^{-1}; v = 16, p= 0.04; Table 3.1). However, in two broods (13% of the clutches) the younger nestling presented a higher Hg concentration than the older nestling (difference: 0.101 ± 0.040 ng g^{-1}). Accordingly, Hg concentrations in body feathers were marginally correlated with body mass (t-test: t = -1.9351, df = 34, p = 0.061) but the latter was not correlated with age (t-test: t = -1.2903; df = 34; p = 0.206). In each owl nest the pooled mean of Hg concentrations in nestlings (barn owl: 0.549 ± 0.435 ng g^{-1}; eagle owl: 0.777 ± 1.12 ng g^{-1}) did not differ from the concentrations in adult moulted flight feathers (barn owl: 0.766 ± 0.504 ng g^{-1}; v = 15, p = 0.23; eagle owl: 1.31 ± 1.41 ng g^{-1}; v = 16, p = 0.16; Table 3.1).

3.4.2 Age-related differences in Hg concentration at the regional level in barn owl and eagle owl

Considering the total sample from barn owl collected in our study area, mean Hg concentrations were 0.514 ± 0.381 ng g^{-1} in nestlings (median: 0.408, range: 0.117–1.65 ng g^{-1}), 0.652 ± 0.755 ng g^{-1} in first year road-killed individuals (median: 0.483, range: 0.054–3.63 ng g^{-1}) and 0.712 ± 0.456 ng g^{-1} in adults, *i.e.* individuals with two or more years (median:

0.558, range: 0.105–1.83 ng g^{-1}; Fig. 3.1). Regarding eagle owl, mean Hg concentrations were 0.819 ± 1.03 ng g^{-1} in nestlings (median: 0.443, range: 0.043–3.46 ng g-1) and 1.30 ± 1.78 ng g^{-1} in adults (median: 0.636, range: 0.145–7.28 ng g^{-1}; Fig. 3.2). Mercury levels showed no significant differences between age classes both in barn owls (F = 1.187, p = 0.31; Table 3.2) and in eagle owls (F = 1.707, p = 0.20; Table 3.2).

Table 3.1 Variations with age in mercury concentration (ng g^{-1}) in owl feathers from nests (mean, standard deviation, median and range). Results from Wilcoxon test for matched samples are given for comparison between siblings (brood) and adults *versus* progeny (nest). Species (Sp): BO – barn owl, EO – eagle owl

Source	Sp	N	Age	Hg concentration	v	p
Brood	BO	13	Younger nestling	0.263 ± 0.189 0.215 [0.099-0.801]	16	0.04
	BO	13	Older nestling	0.555 ± 0.594 0.308 [0.084-2.07]		
Nest	BO	10	Nestlings	0.549 ± 0.435 0.486 [0.135-1.64]	15	0.23
	BO	10	Breeding adults	0.766 ± 0.504 0.561 [0.316-1.83]		
	EO	10	Nestlings	0.777 ± 1.12 0.327 [0.043-3.46]	16	0.16
	EO	10	Breeding adults	1.31 ± 1.41 0.662 [0.113-4.04]		

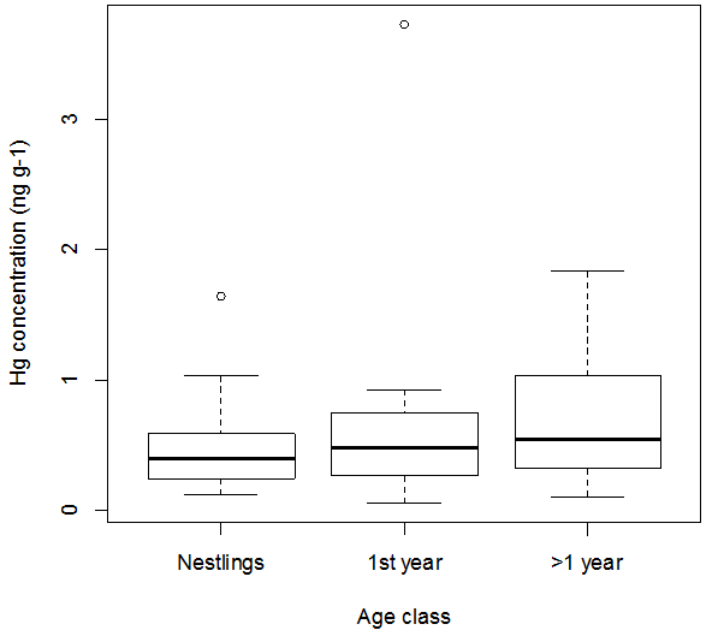

Figure 3.1 Mercury concentration (ng g^{-1}) in barn owl feathers with different age classes. Box and whisker plots show the median, 25% quartiles and range

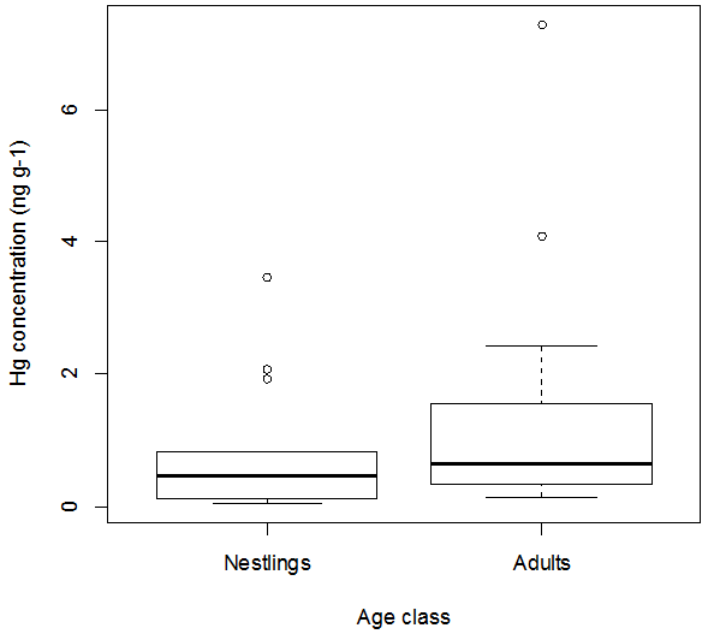

Figure 3.2 Mercury concentration (ng g^{-1}) in eagle owl feathers with different age classes. Box and whisker plots show the median, 25% quartiles and range

Table 3.2 ANOVA results for the analysis of the effect of age on Hg concentration in barn owl feathers collected in nests and road-killed individuals, and in eagle owl feathers collected from nests (unrelated individuals) in south Portugal

	DF	Sum Sq.	Mean Sq.	F	p
Barn owl					
Age	2	1.55	0.773	1.187	0.31
Residuals	57	37.11	0.651		
Eagle Owl					
Age	1	2.64	2.635	1.707	0.20
Residuals	30	46.32	1.544		

3.4.3 Age-related differences in Hg concentration in barn owl feathers collected in nests *versus* road-killed individuals

None of the comparisons between barn owl feather samples from nests and road--killed individuals with regard to age showed significant differences: (1) comparing feathers from nestlings and first year road-killed owls ($t = 0.774$, $df = 32$, $p = 0.45$); (2) comparing feathers from nestlings with adult road-killed owls ($t = 1.966$, $df = 32$, $p = 0.058$); (3) comparing feather of adults and first year road-killed owls ($t = 0.197$, $df = 32$, $p = 0.85$); and (4) comparing a pool of nestling and adult feathers with adult road-killed owls ($t = 1.295$, $df = 32$, $p = 0.20$; Table 3.3).

Table 3.3 Variations with age in mercury concentration (ng g^{-1}) in barn owl feathers from nests and road- -killed individuals (mean, standard deviation, median and range). T-test results are given for comparison between sample types and age classes

	Road-killed individuals	
Feather origin/*age*	*1 year*	*2 years +*
Nests	0.638 ± 0.674 0.520 [0.076–2.78]	0.681 ± 0.453 0.558 [0.105–1.51]
Nestlings only 0.464 ± 0.287 0.390 [0.117–1.03]	t = 0.774, df = 32 p = 0.45	t = 1.966, df = 32 p = 0.058
Nestlings and breeding adults 0.396 ± 0.245 0.333 [0.117–1.03]	t = 0.197, df = 32 p = 0.85	t = 1.295, df=32 p = 0.20

3.5 Discussion

3.5.1 Age effect in Hg concentrations at the territory level in the barn owl and eagle owl

The first laid egg of a clutch most likely contains more Hg than subsequent eggs, corresponding to a decontamination of the female body burden (Becker 1992), and consequently a larger amount of Hg is deposited during embryonic development in older chicks (Becker *et al*. 1994). However, since egg mass represents only 2-8% of a completely grown chick, egg contamination is considered to be negligible (Monteiro and Furness 1995). Instead, variation in age-related trends of Hg concentrations in nestling feathers should depend on the balance between Hg inputs by food intake and dilution by large body mass increments during chick development (Tavares *et al*. 2005). The dilution effect occurs when the mass increase rate exceeds the Hg deposition rate in internal tissues, and thus higher Hg concentrations have been associated to slower growth rates in nestlings (Goutner *et al*.

2001). The combination of an increase in exposure to Hg as nestlings grow, along with an increase in body-size (which may decrease Hg inputs to plumage due to metal dilution into growing internal organs; Thompson *et al*. 1991; Monteiro *et al*. 1995), may determine that Hg accumulation patterns are mostly associated with body mass, and not so much with age. Barn owl nestlings have a bell-shaped curve in mass distribution with age, since there is an overshoot in body mass at ca. 40 days old, followed by a decrease until fledging, at ca. 60 days old (Durant and Handrich 1998). This is caused by a spontaneous decrease of food intake preceding fledging, probably representing an advantage in terms of food resources portioning between siblings with hatching asynchrony – *i.e.* the spontaneous decrease in food intake in older siblings allows for transferring food resources to the younger ones (Durant and Handrich 1998). Also, feather growth seem to be another factor determining Hg concentration in fledglings feathers (Becker *et al*. 1994; Tavares *et al*. 2005). Since the period when chicks may start using their body reserves is concurrent with the completion of 50% feather growth (Durant and Handrich 1998), it is possible that the higher Hg concentrations in older nestlings result from Hg increase in blood due to lipid mobilization (Lewis and Furness 1991; Castro *et al*. 2011). Concluding, siblings sampled at younger ages (before 40 days old) would possibly be in the ascending phase of the body mass curve and therefore Hg concentrations in their body feathers may be under influence of the above mentioned dilution effect (Becker *et al*. 1994; Tavares *et al*. 2005), thus resulting in lower levels of contamination.

Our results suggest that pooling feathers of different nestlings from the same brood, or nestlings and adult moulted feathers from the same nest give similar results, at least in these two owl species. Although some studies have reported differences between nestlings and adults in feather Hg concentrations, these were not always consistent, possibly due to differences across age classes in body weight, prey-size selection and trophic level, resulting in age-related differences in Hg intake or accumulation (Evers *et al*. 2005; Burgess and Hobson 2006; Keller *et al*. 2014). In opposition, our results suggest similarity between age classes in the two sampled owl species, although we do not exclude the possibility that a larger sample size could yield a different output. All considered, in order to reduce possible body mass-effects on Hg concentrations measured at the territory level (particularly in nestlings), we suggest that whenever possible the mean Hg contamination for a given owl

territory should be calculated from more than one individual per nest, regardless of age class.

3.5.2 Age-related differences in Hg concentration at the regional level in barn owl and eagle owl

The barn owl and eagle owl are resident species (Cramp 1985), thus we expected that road-killed individuals were collected near their territory (territorial adults) or during post--fledging dispersal. In resident species both juveniles and adults are contemporaneously subjected to the same background contamination, at the breeding territory. However, adults may have been exposed to different Hg levels during the life history stages, *i.e.* bioaccumulation resulting from Hg intake at natal territory, plus the post-fledging dispersal areas, and the current territory. Both owl species have a complex moult, shading only few feathers each year (Martinez *et al*. 2002; Solheim 2011), therefore we assume some feathers used in the analysis corresponded to firstly shaded feathers, *i.e.* reflecting Hg intake during feather formation while nestlings at the natal territory. However, we have no possibility of determining age for most shed feathers collected in nests for these two species. Regarding road-killed birds, in a sample of 157 feathers from 37 individuals, only 5.8% corresponded to adult flight feathers. Since mean Hg concentration was calculated using 4–5 feathers from each individual, eventual age-effects were most likely diluted by the calculation (Roque *et al*. 2016).

Age-related differences in Hg concentrations are frequently associated with differences in Hg intake between nesting and wintering grounds in migratory species (Monteiro *et al*. 1995; Becker *et al*. 2002). However, rather than geographically dependent, age-related differences may result mostly from the time elapsed between exposure and sample collection, which reflects a time-trend in bioaccumulation. The lack of differences in Hg contamination with age in barn owls and eagle owls most likely results from both species being resident and having similar diet in adults and nestlings, which should result in similar Hg concentrations in blood of both age classes at time of feather growth. In the case of first year road-killed owls, feathers were formed in the nest; therefore, Hg concentration will reflect local contamination at the natal site, but not necessarily at the collection site.

Although a few barn owl feather samples may not be considered independent as they belong to adults and juveniles from the same nest-site, we assumed that the contribution of increasing sample size to be more relevant than increasing the probability of type II error. The validity of the results obtained for the barn owl was supported by the concordant results found for eagle owls, which included only un-related individuals.

3.5.3 Age-related differences in Hg concentration in barn owl feathers collected in nests *versus* road-killed individuals

Our results suggest that feathers from road-killed barn owls can be collected irrespectively of age, since Hg concentrations are comparable with those measured in feathers collected in owl territories in the same area. First year barn owls were more frequently found dead on roads than adults, thus our comparisons were mostly based on Hg concentrations in feathers formed in the first three months after hatching. In practice, feathers from nestlings and first-year owls represent the contamination at the natal site, therefore, Hg concentration should be influenced mostly by site-dependent effects (local contamination and owl diet) and not by age-related effects. In an opportunistic sampling scenario, in which there is a higher likelihood of sampling first-year feathers, Hg concentrations in barn owl feathers from nests and road-killed birds will most likely represent environmental variation. Barn owls are known to disperse mostly within a distance of 50 km from their natal sites (Bunn *et al*. 1982; de Bruijn 1994) and in our study area maximum recovery distance was ca. 60 km (I. Roque, A. Marques, R. Lourenço, J.E. Rabaça unpublished data). Breeding adults are probably road-killed inside their home range, estimated to be 763 ± 665 ha in South Portugal (Grilo *et al*. 2012). Accordingly, we can roughly estimate an area around the sampling site that is characterized by the Hg concentrations found in road killed barn owl feathers, which is likely to be more restricted in older birds. This error margin should be less relevant as many monitoring programs have a broad scale approach. Moreover, the effect of external contamination in Hg concentrations in barn owl feathers seems negligible, considering we found no differences in Hg levels between nest-formed feathers with different age (*i.e.* a proxy of the time feathers were exposed to external contamination).

3.6 Conclusions

The use of owl feathers for biomonitoring Hg is challenging because of the possible confounding effects, including feather mass (Roque *et al*. 2016) and age. In barn owls and eagle owls, Hg levels do not seem to be largely affected by the age of the sampled individuals. However, in an opportunistic sampling scenario feathers from different age classes can also be associated to spatial variation, since they may represent contamination in different locations, even if no differences in Hg concentration are found. Feathers from nestlings only represent Hg contamination at the natal site, which coincides with the sampling site. Feathers from first year road-killed birds represent Hg contamination at the natal site, which may not coincide with the sampling site. Finally, Hg concentration measured in adult feathers collected in nest sites and in feathers collected from most adult road-killed birds should represent contamination at sampling site, but may be influenced also by contamination in other locations where the individuals spent time in earlier life stages (*e.g.* natal site, dispersal areas). Consequently, using as many feather samples as possible seems the most effective way of obtaining a reliable estimate of local Hg contamination, by controlling the variation resulting from confounding effects.

4

Effect of diet, habitat and industrial emissions in biota-soil mercury accumulation factors in the barn owl (*Tyto alba*)

This chapter was based on the manuscript in preparation:

Roque I, Lourenço R, Marques A, Coelho JP, Cruz N, Pereira E, Roulin R, Rabaça JE. Effect of diet, habitat and industrial emissions in biota-soil mercury accumulation factors in the barn owl (*Tyto alba*)

4 Effect of diet, habitat and industrial emissions in biota-soil mercury accumulation factors in the barn owl (*Tyto alba*)

4.1 Abstract

The toxicological impact of Hg depends on how it is distributed between exposure medium and biota. The relation between environmental levels and Hg contamination in top predators is rarely addressed in toxicological studies because of the complex trophic effects in the bioaccumulation of pollutants along food chains. We explored the biota-soil accumulation factors (BSAF), in order to inspect the influence of diet, habitat and industrial emissions in Hg dynamics in barn owl (*Tyto alba*) territories. We measured Hg concentrations of 69 soil samples and 56 barn owl feathers from 23 nest sites. Median BSAF was 36.8 times (5.81–938 times) with a large variability in the relation between Hg concentrations in soil and feathers among nest sites, suggesting local variation in the factors affecting bioaccumulation. There was no clear effect of diet on BSAF. Biological Hg exposure seems to be detached from industrial Hg emissions, which also did not show a relation with soil concentrations.

Agricultural land uses seemed to best explain bioaccumulation in the barn owl, particularly, permanently irrigated and heterogeneous agricultural areas. These land uses were associated with lower Hg concentrations in soil and feathers, which may be cumulatively influenced by different factors, probably related with watering regime and landscape heterogeneity. Lower Hg concentrations in irrigated soils may be associated with lower Hg agricultural input and/or faster Hg decontamination. Habitat may influence Hg bioaccumulation in barn owls by determining Hg levels in prey but also by affecting diet composition in barn owls (via prey abundance and availability). Land use may produce effects in both concentrations in feathers and soil but still not affect BSAF (*i.e.* show no obvious trend in Hg bioaccumulation from soil to feathers). Thus, BSAF should be analyzed together with Hg concentrations in biota and soil. Biota-soil accumulation factors in raptors seem to be useful indicators of habitat-related contaminant dynamics in terrestrial environment.

Keywords: Barn owl, bioaccumulation, diet effects, industrial emissions, mercury, soil contamination

4.2 Introduction

Mercury is a widespread pollutant that is likely to increase globally through atmospheric deposition (Costa *et al*. 2006; Windham-Myers 2014). Terrestrial soils are the largest Hg reservoirs and the risk of rising concentrations of this metal threats ecosystems because of the formation of the toxic methylmercury (MeHg; Amos *et al*. 2013; Krabbenhoft and Sunderland 2013). Half of the atmospheric Hg derived from anthropogenic emissions is removed by local and regional-scale processes such as dry deposition and precipitation scavenging of the scarce and short-resident atmospheric particulate Hg (Monteiro *et al*. 1999). Consequently, high spatial variation in Hg concentrations is expected in terrestrial ecosystems. The potential for exposure of terrestrial biota to a chemical depends on a complex interaction of several factors, including the chemical's environmental fate, the physical and chemical characteristics of the chemical and the soil, and a series of abiotic and biotic processes (such as hydrolysis, photolysis, biodegradation, soil adsorption and mobility, volatilization from water or soil, biodegradation, etc.), which ultimately influence bioavailability (Hoke *et al*. 2016). Bioaccumulation is the process by which contaminants as Hg are absorbed in organisms and undergo enrichment in relation to the environment, as a result of all uptake and loss processes, *e.g.* dietary and environmental uptake, feacal egestion, transfer to offspring, metabolic biotransformation and growth dilution (Arnot and Gobas 2006; Jorgensen 2010; Borgå *et al*. 2012). Bioaccumulation comprises the more specific processes of bioconcentration (*i.e.* direct portioning of chemicals between the medium and the organism) and biomagnification (*i.e.* uptake from the diet leading to higher concentrations in the organism than in its prey, resulting in increased chemical concentration with higher trophic position in the food chain; Gobas and Morrisson 2000; Jorgensen 2010).

Bioaccumulation is often reported by biota-soil accumulation factors (BSAFs), which in a broad context are interchangeably referred to as bioconcentration factors (BCFs; Walker *et al*. 2012; Pereira *et al*. 2006; Falusi and Olanipekun 2007) and bioaccumulation factors (BAFs; *e.g.* ; Mason and Lawrence 1999; Hoffman *et al*. 2003). Bioaccumulation factors are

typically measured under field conditions that include the total chemical concentration in the water phase (for aquatic environments), but also in sediment or soil (McGeer *et al*. 2003; Arnot and Gobas 2006). Some authors consider BCFs to describe the exposure exclusively from the abiotic environment, excluding food uptake (Gobas *et al*. 2009; Jorgensen 2010). In this sense, BCF is considered as laboratory-derived and BAF as field-derived (McGeer *et al*. 2003). Additionally, some authors assume as BAFs what others assume as biomagnification factors (BMFs), by calculating the ratio between concentrations in an organism and concentrations in its food (*e.g.* Jongbloed *et al*. 1994; Dietz *et al*. 2000). We considered BSAF (concentration in biota divided by concentration in the medium) as an unit-less number between zero and infinity (Hoffman *et al*. 2003) representing a single-compartment model that predicts partitioning between exposure medium and biota, showing the potential toxicological impact of Hg (McGeer *et al*. 2003).

Raptors (birds of prey and owls) are among the groups of vertebrates exposed to the highest levels of toxicants due to bioaccumulation and biomagnification along food chains (Lindberg and Odsjö 1983; Lourenço *et al*. 2011). As a result, raptors are broadly used for biomonitoring metals like Hg in terrestrial environments (Espín *et al*. 2016). Since many species are widespread, territorial and mostly sedentary, metal concentrations in these birds may be related to metal contamination in a source area, allowing spatial variation assessment (Esselink *et al*. 1995; Jager *et al*. 1996; Becker 2003; Evers *et al*. 2005). Given the ethical issues involved in using these organisms as biomonitors, raptor feathers are often selected as non-invasive monitoring tools (Solonen and Lodenius 1990; Dauwe *et al*. 2003; Martínez *et al*. 2012; Gómez-Ramírez *et al*. 2014). Deposition in feathers is the main excretory pathway for Hg (Lewis and Furness 1991) and its concentrations reflect endogenous deposition of the blood-circulating metal during the feather growth phase (Denneman and Douben 1993; Dauwe *et al*. 2003). After full formation, the blood vessels irrigating follicles undergo atrophy and Hg remains stable in the feather, mostly in the form of MeHg (Furness *et al*. 1986; J. Burger 1993).

We studied the effect of diet composition in BSAFs in a medium sized opportunistic predator that typically feeds in open farmlands – the barn owl (*Tyto alba*; Bunn *et al*. 1982; Roulin 2002). This species has a widespread distribution in Portugal, being more abundant south to the Tagus River (Lourenço *et al*. 2015). In the Tagus valley the species has an

estimated population density between 0.05 and 0.07 pairs km^{-2}, which compared to other regional estimates suggests a great importance of the area for the barn owl (Roque and Tomé 2004). Moreover, the Tagus estuary is a relevant hunting area for the species during post-fledging dispersal, with abundance index reaching 2.5 owls km^{-1} in mid-autumn (Tomé and Valkama 2001).

The Tagus Estuary is one of the largest wetlands in Europe (320 km^2) and has been classified as one of the most polluted with Hg (Figuéres *et al*.1985), with estimated inputs of 5 t $year^{-1}$ in the 1980s (Canário 2004). High Hg concentrations have been detected in physical samples like suspended particles (Figuéres *et al*.1985; Canário *et al*. 2003), but also in organisms like algae (Ferreira 1988) and fish (Lima *et al*. 1982). A study with an aquatic bird, the black-winged stilt (*Himantopus himantopus*), also revealed elevated Hg contamination in the Tagus compared to other Portuguese estuaries (up to 3.4 ± 1.5 µg g^{-1} in feathers; Tavares *et al*. 2004). Nevertheless, there is no information on Hg contamination in terrestrial ecosystems in the vicinity of the estuary. In this study we analyzed the potential toxicological impact of Hg contamination and interpreted accumulation mechanisms in terrestrial ecosystem by comparing Hg concentrations in barn owl feathers with those in soil in the southwest Tagus basin, and in Sado and Guadiana basins. Our main goals were (1) to characterize bioaccumulation in a terrestrial predator associated to agricultural landscapes; (2) to identify spatial patterns in bioaccumulation, (3) to assess the effect of diet composition and diversity in Hg bioaccumulation in the barn owl, and (4) to assess the effect of habitat and main industrial Hg sources in bioaccumulation.

4.3 Materials and methods

4.3.1 Study area

The study area is located in South Portugal, comprising the southwest Tagus basin and the western part of Sado and Guadiana basins (Fig. 1). The Tagus area includes the Tagus estuary, enclosed by north by the metropolitan area of Lisbon. The climate is Mediterranean with hot and dry summers and mild winters, and the rain fall is generally concentrated in the cold season. Landscape is characterized by plain or undulating lowlands dominated by agricultural land uses (48%) in Tagus and by forests (55%) in Sado and Guadiana basins. Main

agricultural land uses are permanently irrigated cultures (19%) in the Tagus area and non--irrigated arable land (23%) in Sado and Guadiana (European Environment Agency 2007). Some of the industries located in the study area report releases above the threshold for Hg in air (10 kg $year^{-1}$; Regulation No. 166/2006 of the European Parliament and of the Council). These include coal-burning power plant located in the municipality of Abrantes (A); a chemical and metal industrial complex in Barreiro (B); a thermal power station (fuel-oil--powered, closed in 2012), a cement industry and a manufacture of pulp and paper in Setúbal (C); and the most potent thermal plant (coal-powered) in Portugal, and also an industrial complex for manufacture of refined petroleum products in Sines (D). These industries are monitored under the European Pollutant Release and Transfer Register (E-PRTR; European Commission 2006).

4.3.2 Sample collection

Samples were collected at 23 barn owl territories located in the southern part of the Tagus basin (n = 18) and western part of Sado and Guadiana basins (n = 5) (Fig. 4.1). A total of 56 feather samples (2.4 ± 1.1 feathers per nest) were obtained between 2010 and 2012, either plucked from the bodies of live barn owls or found shed. In the same visits to nests, three superficial soil samples with 100 g were collected. Additionally, barn owl diet was characterized using a total of 1 188 pellets collected in nests (51.5 ± 25.0 pellets per nest). All feathers and soil samples were stored in transparent plastic bags until chemical analysis. Pellets were freeze stored in plastic bags until diet analysis.

4.3.3 Diet analysis

Prey were identified whenever possible to species level using identification keys for bones. The minimum number of individuals in each prey category was determined for every diet sample. For each sampling site we calculated the percentage in terms of number of individuals (PON; number of individuals of the prey group divided by the total number of individuals in the sample) for each prey category for the two trophic levels considered: primary consumers (*Apodemus sylvaticus* and *Microtus* spp.) and secondary consumers (*Crocidura* spp., *Talpa occidentalis*, *Mus* spp. and *Rattus* spp.). Diet diversity was estimated by Shannon's diversity index calculated with logarithm of base 10 at the genus taxonomic level for rodents, and at class taxonomic level for other prey types.

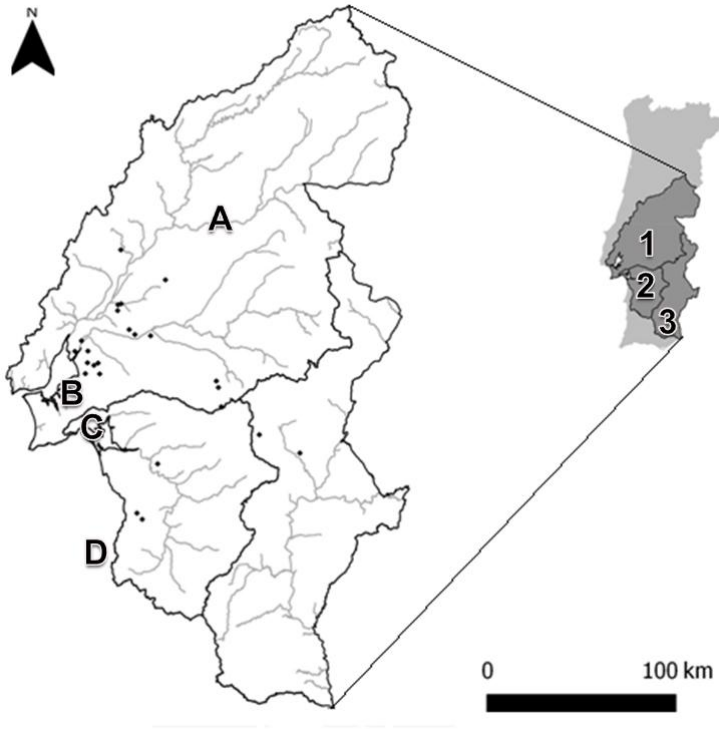

Figure 4.1 Study area and location of sampling sites (black dots). 1 – Tagus basin, 2 – Sado basin, 3 – Guadiana basin; Hg sources: A – Abrantes (coal-burning power plant), B – Barreiro (metal industrial complex), C – Setúbal (cement industry, pulp and paper manufacturer); D – Sines (thermal plant, refined petroleum products manufacturer)

4.3.4 Mercury analysis

Soil samples were sieved to remove coarse particles. Total Hg concentrations were measured by thermal atomization atomic absorption spectroscopy, following the same method as described in Roque *et al*. (2016). The quality control of results was assured using reference material (TORT-2), which delivered an accuracy of 10 % with 95 % confidence intervals. Reproducibility was inspected by succeeding measurements on the same sample, which delivered standard deviations lower than 5 %; and detection limit was 0.01 ng for a 100 mg sample. Hg concentrations are presented in ng g^{-1} on a fresh weight basis.

4.3.5 Statistical analysis

We calculated BSAF by dividing the Hg concentration in the barn owl for each territory (mean value for all feathers collected in the nest site) by the Hg concentration in

the soil of that territory (mean value for all soil samples in the territory). As explanatory variables we used (1) diet variables (PON of four prey groups - *Microtus* spp, *Apodemus sylvaticus*, *Mus* sp., insectivores; and diet diversity); (2) dominant land use (agricultural, forest or mixed) in the barn owl territory (2-km buffer centred in the nest site); (3) area of the four main agricultural land uses in each territory (2-km buffer) – permanently irrigated land, non-irrigated arable land, heterogeneous agricultural areas (*i.e.* juxtaposition of small parcels of diverse annual crops, pasture and/or permanent crops, and areas principally occupied by agriculture, interspersed with natural areas; Néry 2007), and permanent cultures; (4) distance of each nest site to the four main sources of industrial emissions (A, B, C, D).

Data were inspected for outliers and distribution normality of the variables (*e.g*. Quinn and Keough 2002), and a logarithmic transformation was applied to BSAF, and Hg concentrations in feathers and soil. Linear regression models were used to evaluate the variation of BSAF with diet variables, land use variables and distance to the four main sources of industrial emissions. Information-theoretic methods were used for multimodel inference based in AICc values – second-order Akaike's information criterion (Burnham and Anderson 2002). We considered an AICc difference (ΔAICc) of 2.0 to define the set of competing models (Burnham and Anderson 2002). We used model diagnostic plots to validate model results. All statistical analyses were conducted using R software 3.1.1 (R Core Team 2014) with the package MuMIn (Barton 2014).

4.4 Results

4.4.1 Mercury bioaccumulation in the barn owl

Mean Hg concentration was 0.022 ± 0.026 ng g^{-1} (median: 0.014 ng g^{-1}; range: 0.001–0.104 ng g^{-1}) in soil and 0.630 ± 0.497 ng g^{-1} (median: 0.414 ng g^{-1}, range 0.117–1.87 ng g^{-1}) in barn owl feathers, resulting in a mean BSAF of 93.7 ± 192 times (median: 36.8, range: 5.81–938 times). There was a great variation in BSAFs in barn owls (from only 6 to more than 900 times more Hg in owls than in the soil), with similar median values in Tagus and Sado-Guadiana basins (45.4 and 36.8, respectively; Table 4.1). Nevertheless, Hg concentrations in both soils and barn owl feathers were higher in Sado-Guadiana (0.032 and 1.16, respectively;

Table 4.1), suggesting that higher environmental contamination with Hg produces higher contamination in the barn owl and the relation between Hg levels in the environment and in feathers may be comparable between regions with different background contamination. However, at a smaller-scale analysis, territories located along the Sorraia River (n = 3), a tributary of Tagus River, showed the highest BSAF values in BSAFs in this basin, and also a great range (Fig. 4.2). Barn owl territories in Sorraia showed a relatively low Hg concentration in soil (Fig. 4.3) and a relatively high Hg concentration in barn owl feathers (Fig. 4.4). A different pattern was patent in Alviela (n = 1), with the lowest BSAFs and the highest Hg concentration in soil, and in the range of lower values of Hg concentration for the basin in feathers. High variation in Hg bioaccumulation at small scale (*i.e.* nest site) seemed strongly influenced both by variations in environmental levels and by factors affecting Hg concentrations in feathers.

Table 4.1 Regional variation in Hg concentrations (ng g^{-1}) in soil and feathers, and respective biota-soil accumulation factors (mean ± standard deviation, median, range, and linear regression results)

Basin	Soil*	Feathers*	Biomagnification
Tagus	0.013 ± 0.012	0.479 ± 0.341	107 ± 216
(n = 18)	0.009 [0.001–0.042]	0.362 [0.117–1.26]	45.4 [6.22–938]
Sado-Guadiana	0.053 ± 0.038	1.17 ± 0.626	44.7 ± 47.5
(n = 5)	0.032 [0.015–0.105]	1. 16 [0.488–1.87]	36.8 [5.81–121]
Difference between river basins	F = 11.14; p = 0.003	F = 8.902; p = 0.007	F = 0.984; p = 0.332

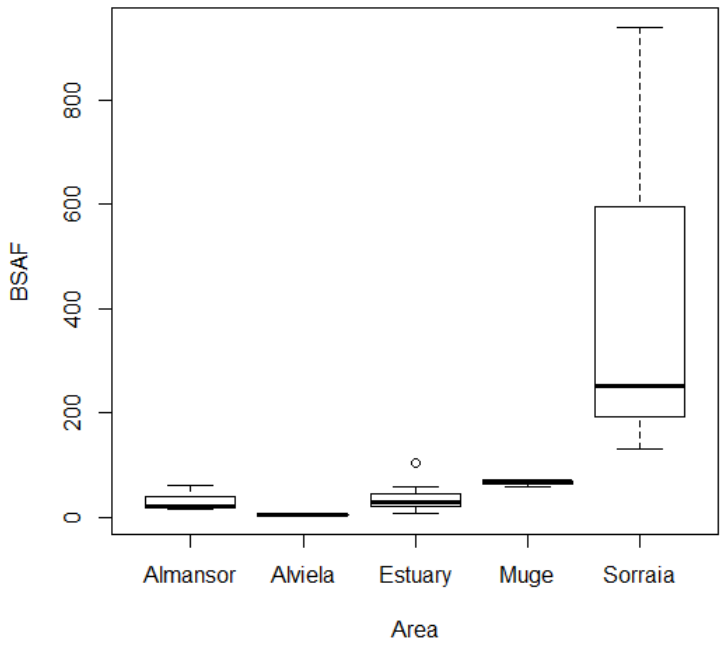

Figure 4.2 Variation in bioaccumulation factors (BSAF) in barn owl in five areas of the Tagus basin. Box and whisker plots show the median, 25% quartiles and range

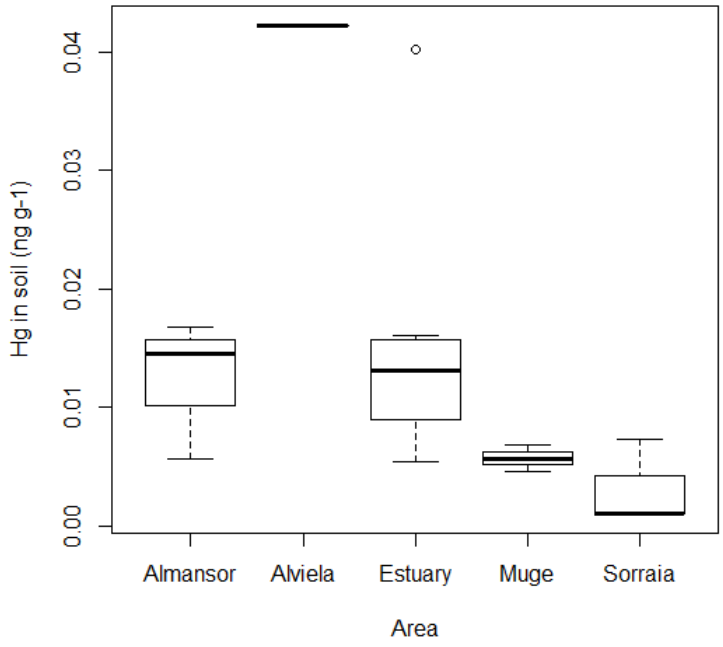

Figure 4.3 Variation in mercury concentrations (ng g^{-1}) in soil from barn owl territories in five areas of the Tagus basin. Box and whisker plots show the median, 25% quartiles and range

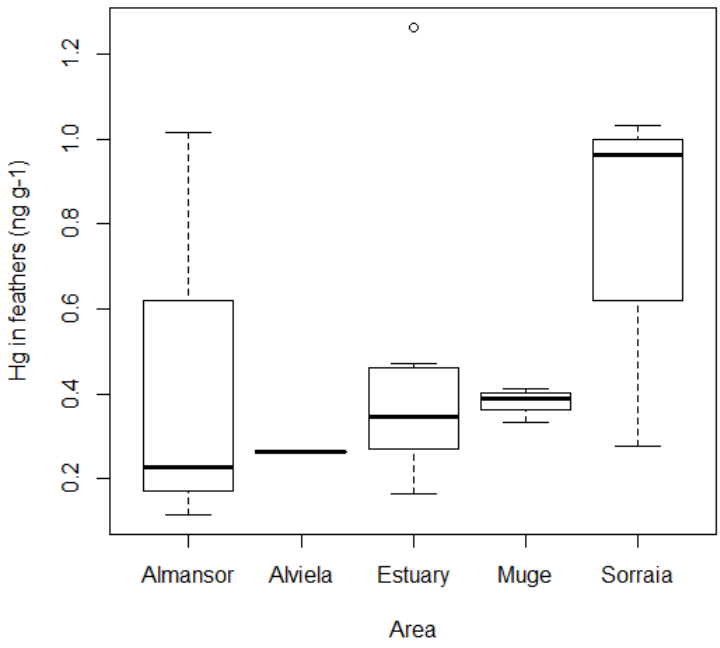

Figure 4.4 Variation in mercury concentrations (ng g^{-1}) in barn owl feathers in five areas of the Tagus basin. Box and whisker plots show the median, 25% quartiles and range

4.4.2 Effect of diet composition and diversity in mercury bioaccumulation

Barn owl diet in south Portugal was almost evenly composed by primary (47.2 ± 20.4%) and secondary consumers (53.9 ± 19.2%), with a dominance of *Mus* spp. (30.5 ± 19.9%) among the secondary consumers, and *Apodemus sylvaticus* (23.9 ± 12.1%) and *Microtus* spp. (21.8 ± 12.7%) among the primary consumers. Mean diet diversity was 0.586 ± 0.119 (median: 0.614, range: 0.219–0.730). Two models were included in the set of best models, the random effects model and the model including the variable diet diversity (Table 4.2). Our data supported best the random effects model (w_i = 0.31) compared to the models testing the effects of diet diversity (w_i = 0.19), prey trophic level (w_i = 0.08) and main prey types (w_i = 0.08 and 0.09; Table 4.2). The evidence ratio for the two best models indicated that the empirical support for the random effects model was 1.6 times that of the model including the variable Shannon diversity index. Therefore, variations in barn owl diet do not have a considerable influence in BSAFs. Although not strongly supported by our data, greater diet diversity could be associated with lower bioaccumulation in the barn owl (Table 4.3).

Diet diversity was similar between basins (linear regression: F = 0.796, p = 0.382) and frequency of insectivores was the only diet-related variable which differed between Tagus and Sado-Guadiana basins (linear regression: F = 0.51, *p* = 0.019).

Table 4.2 Information-theoretic model selection results for the analysis of the effect of diet diversity (Shannon index), prey trophic level (primary and secondary consumers) and main prey types (Insectivorous, *Mus* spp., *Apodemus sylvaticus* and *Microtus* spp.) in biota-soil accumulation factors in the barn owl

Model	df	Log-likelihood	AICc	ΔAICc	w_i
Null model	2	-37.27	79.13	0.00	0.31
Shannon diversity	3	-36.42	80.10	0.97	0.19
Microtus spp.	3	-37.12	81.50	2.37	0.09
Apodemus sylvaticus	3	-37.18	81.63	2.49	0.09
Secondary consumers	3	-37.25	81.77	2.64	0.08
Mus spp.	3	-37.27	81.80	2.66	0.08
Insectivores	3	-37.27	81.80	2.66	0.08
Primary consumers	3	-37.27	81.80	2.66	0.08

Table 4.3 Model results for the analysis of the effect of diet diversity on BSAFs in the barn owl

	Coefficient	SE	z	p
Intercept	5.313	1.317	4.034	0.0006
Diet diversity	-2.795	2.205	-1.267	0.219

4.4.3 Habitat-related effects in mercury bioaccumulation

The information-theoretic analysis of the effects of habitat on BSAFs showed that our data supported best the model with heterogeneous agricultural areas (w_i = 0.70; evidence ratio to second best model = 3.3; Table 4.4). The second best model, which included the variable permanent crops, received considerably less support, with a probability (w_i) of 0.21 of being the best model (ΔAICc = 2.44). These results suggest that Hg bioaccumulation in the barn owl is higher in territories with a greater area occupied by heterogeneous agriculture

(Table 4.5). To a lesser extent, Hg bioaccumulation seemed lower in territories with more area occupied by permanent crops.

Regarding Hg concentrations in soil, our data supported best the model with permanently irrigated areas (w_i = 0.49; evidence ratio to second best model = 2.6; Table 4.4). Some support was also set to the model with heterogeneous agricultural areas, with a probability of 0.19 of being the best model (ΔAICc = 1.92). These results suggest that Hg concentration in soil is lower in barn owl territories with morearea occupied by permanently irrigated agriculture and to a lesser extent with more area of heterogeneous agriculture (Table 4.5). For Hg concentrations in barn owl feathers, the model with permanently irrigated areas (w_i = 0.46; evidence ratio to second best model = 3.1; Table 4.4) was best supported by our data. The models with permanent crops and heterogeneous agricultural areas also received some support, each with a probability of 0.15 of being the best model (ΔAICc = 2.19 and 2.30, respectively). These results suggest that Hg concentrations in barn owl feathers are lower in territories with more area occupied by permanently irrigated agriculture. Although with a smaller effect, Hg concentrations in barn owl feathers also seem to be lower in territories with more area with permanent crops and higher in territories with more area of heterogeneous agriculture (Table 4.5).

Overall, the models with variables non-irrigated arable land and main land use (categorical) were lightly supported by our data. This seems to indicate that the dominance of agricultural or forest areas in barn owl territories has a small effect in Hg contamination in the species, suggesting that variations in Hg concentrations in soil and in feathers, thus in bioaccumulation, are most likely influenced at a smaller-scale by agricultural land uses.

Table 4.4 Results of information-theoretic model selection for the analysis on the effect of dominant land use and four agricultural land uses in biota-soil accumulation factors, and in mercury concentrations in soil and in barn owl feathers

Model	df	Log-likelihood	AICc	ΔAICc	w_i
Bioaccumulation					
Heterogeneous agric. areas	3	-33.37	73.99	0.00	0.70
Permanent crops	3	-34.59	76.44	2.44	0.21
Null model	2	-37.27	79.13	5. 14	0.05
Perman. irrigated areas	3	-37.10	81.47	7.48	0.02
Non-irrigated arable land	3	-37.16	81.58	7.59	0.01
Dominant land use	4	-36.16	82.55	8.55	0.01
Hg in soil					
Perman. irrigated areas	3	-32.81	72.89	0.00	0.49
Heterogeneous agric. areas	3	-33.77	74.81	1.92	0.19
Null model	2	-35.23	75.06	2.17	0.17
Permanent crops	3	-34.47	76.19	3.30	0.09
Non-irrigated arable land	3	-35.23	77.72	4.83	0.04
Dominant land use	4	-34.82	79.87	6.98	0.01
Hg in feathers					
Perman. irrigated areas	3	-22.69	52.64	0.00	0.46
Permanent crops	3	-23.78	54.83	2.19	0.15
Heterogeneous agric. areas	3	-23.84	54.94	2.30	0.15
Null model	2	-25.51	55.61	2.98	0.10
Dominant land use	4	-22.84	55.90	3.26	0.09
Non-irrigated arable land	3	-25.10	57.47	4.83	0.04

Table 4.5 Averaged-model results for the analysis on the effect of dominant land use and four agricultural land uses in biota-soil accumulation factors, and in mercury concentrations in soil and in barn owl feathers

Fixed effect	Estimate	SE	Adjusted SE	Z	p	Relative importance
Bioaccumulation						
Intercept	3.232	0.633	0.644	5.018	0.000	
Heterogeneous agric. areas	0.814	0.279	0.296	2.745	0.006	0.70
Permanent crops	-0.704	0.300	0.318	2.213	0.027	0.21
Perman. irrigated areas	0.171	0.311	0.331	0.517	0.605	0.02
Non-irrigated arable land	0.145	0.329	0.350	0.416	0.677	0.02
Dominant (forest)	0.544	0.561	0.598	0.909	0.363	0.02
Dominant (mixed)	1.169	0.950	1.011	1.157	0.247	0.02
Hg in soil						
Intercept	-4.105	0.418	0.433	9.483	0.000	
Perman. irrigated areas	-0.574	0.259	0.275	2.089	0.037	0.49
Heterogeneous agric. areas	-0.479	0.284	0.302	1.589	0.112	0.19
Permanent crops	0.358	0.298	0.316	1.133	0.257	0.09
Non-irrigated arable land	0.024	0.303	0.321	0.073	0.942	0.04
Dominant (forest)	0.157	0.530	0.564	0.279	0.781	0.01
Dominant (mixed)	-0.633	0.896	0.953	0.664	0.507	0.01
Hg in feathers						
Intercept	-0.613	0.342	0.350	1.752	0.080	
Perman. irrigated areas	-0.403	0.167	0.177	2.277	0.023	0.46
Permanent crops	-0.346	0.187	0.199	1.737	0.082	0.15
Heterogeneous agric. areas	0.334	0.185	0.196	1.707	0.088	0.15
Dominant (forest)	0.701	0.315	0.335	2.092	0.037	0.09
Dominant (mixed)	0.536	0.532	0.566	0.946	0.344	0.09
Non-irrigated arable land	0.169	0.195	0.207	0.816	0.414	0.04

4.4.4 Effect of potential industrial sources in mercury bioaccumulation

Our data supported best the null model compared to the models testing the effects of distance to main industrial sources in BSAF (w_i = 0.44), in Hg concentrations in soil (w_i = 0.37) and in Hg concentrations in barn owl feathers (w_i = 0.18; Table 4.6). The evidence ratio for the two best models indicated that the empirical support for the null model was 2.8 times higher for BSAF, 1.5 for Hg concentrations in soil and 1.0 for Hg concentrations in feathers. These results suggest that the distance of barn owl territories to main industrial sources could not clearly explain the variation in BSAFs, and neither on Hg levels in soil or feathers. Nevertheless, among the four contamination sources, distance to Abrantes power plant is the one that better explains the variations in Hg concentrations in soil and feathers, which decrease with distance (Table 4.7).

Table 4.6 Results of information-theoretic model selection for the analysis on the effect of distance to the main industrial sources of atmospheric Hg in biota-soil accumulation factors, and in mercury concentrations in soil and in barn owl feathers

Model	df	Log-likelihood	AICc	ΔAICc	w_i
Bioaccumulation					
Null model	2	-37.27	79.13	0.00	0.44
Barreiro	3	-36.95	81.16	2.03	0.16
Setúbal	3	-37.00	81.26	2.13	0.15
Sines	3	-37.14	81.55	2.41	0.13
Abrantes	3	-37.23	81.72	2. 58	0.12
Hg in soil					
Null model	2	-35.23	75.06	0.00	0.37
Abrantes	3	-34.31	76.88	0.82	0.25
Sines	3	-34.85	76.96	1.90	0.14
Barreiro	3	-34.93	77. 12	2.05	0.13
Setúbal	3	-35.13	77.53	2.47	0.11

Table 4.6 Results of information-theoretic model selection for the analysis on the effect of distance to the main industrial sources of atmospheric Hg in biota-soil accumulation factors, and in mercury concentrations in soil and in barn owl feathers (continued)

Model	df	Log-likelihood	AICc	ΔAICc	w_i
Hg in feathers					
Null model	2	-25.51	55.61	0.00	0.35
Abrantes	3	-24.22	55.71	0.09	0.34
Setúbal	3	-25.36	57.98	2.37	0.11
Sines	3	-25.38	58.03	2.42	0.11
Barreiro	3	-25.50	58.26	2.64	0.09

4.5 Discussion

4.5.1 Mercury bioaccumulation in the barn owl

Mercury bioaccumulation in barn owl feathers is much higher than in most reported organisms (Table 4.7). In general, BSAFs are calculated for organisms in which the main contamination source is absorption from the medium, being therefore generally applied to aquatic organisms (McGeer *et al*. 2003; Arnot and Gobas 2006). For organisms on or after the second level in the food web the uptake occurs mainly via the food, therefore BMFs or biomagnification trophic factors (BTFs) are instead calculated, in order to include biomagnification as a factor of bioaccumulation (Walker *et al*. 2010; Borgå *et al*. 2012). Nevertheless, BSAFs at high levels in terrestrial organisms can help to find organisms susceptible to bioaccumulation and to interpret the mechanism of accumulation (UNEP 2011). The relatively high values of BSAF in the barn owl compared with those recorded in terrestrial invertebrates which are closer to the bottom of its food web (up to 303 times the maximum BSAF in earthworms; Burton *et al*. 2006; Zhang *et al*. 2009), and the closeness to values detected in Hg-tolerant plants in a contaminated mining area (up to 0.75 times BSAF in *Rumex induratus*, Moreno-Jiménez *et al*. 2006) raise concern on the possible barn owl suceptibility to environmental Hg contamination. These comparisons are merely indicative because they refer to varying biotic and abiotic exposure conditions, but they are here considered to frame the barn owl in the known range of field-derived BSAFs.

We observed overall concurrent patterns in Hg concentrations in soil and feathers, *i.e.* suggesting that areas with higher background Hg contamination in general result in higher contamination in barn owls. However, at a more local scale, the relation between Hg contamination in soils and in barn owls was not always as clear. Soils with low Hg contamination may be associated with high Hg concentrations in feathers (*e.g.* Sorraia), and soils with high Hg concentrations may be associated with concentrations is feathers in the range of the values for the overall area (*e.g.* Alviela). This variability suggests that factors affecting bioaccumulation are diverse and probably entangled. Consequently, Hg concentrations in soil may be a poor indicator of potential local effects in wildlife, chiefly if results are derived from small sample sizes. This variation in bioaccumulation may occur at inter-compartment transfer and/or at food web transfer. Differences in BSAFs may be affected by (1) variations in the bioaccessibility of Hg, because higher proportions of MeHg may not always occur in the most contaminated sediments (Canário *et al*. 2005) and (2) variations in barn owl diet, because this is the main source of Hg intake to the organism (Lewis and Furness 1991; Monteiro and Furness 1997; Thompson *et al*. 1998).

Table 4.7 Biota-soil accumulation factors (BSAF) for mercury calculated as concentration in biota divided by concentration available in sediments (aquatic organisms) or soil (terrestrial organisms)

Organism	Species	BAF	Sampling area	Source
Aquatic				
Macrophytes	*Codium amplivesciculatum*	0.136	Gulf of California (USA)	(Green-Ruiz *et al*. 2005)
	Enteromorpha clathrata	0.184		
	Gracilaria clathrata	0.130	Gulf of California (USA)	(Green-Ruiz *et al*. 2005)
	Ulva lactuca	0.079		
	Ulva sp.	0.14	Sado Estuary (Portugal)	(Lillebo *et al*. 2011)
Aquatic				
Plants	*Halimione portulacoides*	0.10–2.20	Portuguese coast	(Válega *et al*. 2008)
		0.16-0.22	Ria de Aveiro (Portugal)	(Castro *et al*. 2009)
	Triglochin maritima	0.13–0.34		
	Juncus maritimus	0.05-0.15		

Table 4.7 Biota-soil accumulation factors (BSAF) for mercury calculated as concentration in biota divided by concentration available in sediments (aquatic organisms) or soil (terrestrial organisms) (continued)

Organism	Species	BAF	Sampling area	Source
Aquatic				
	Juncus maritimus	0.05-0.15		
	Sarcocornia perennis	0.05-0.07		
Worms	*Hediste diversicolor*	0.23	Sado Estuary (Portugal)	(Lillebo *et al*. 2011)
		0.00–0.08	Portuguese coast	(Cardoso *et al*. 2009)
Molluscs	*Scrobicularia plana*	0.40–1.14		(Coelho *et al*. 2014)
		0.00–0.03		(Cardoso *et al*. 2009)
	Chione subrugosa	0.086	Gulf of California (USA)	(Green-Ruiz *et al*. 2005)
Molluscs	*Crassostrea gigas*	0.314		
Benthic org.	Not specified	0.03–2.14[a)]	Bay of Biscay (France)	(Monperrus *et al*. 2005)
Crabs	*Carcinus maenas*	0.32–8.20	Ria de Aveiro (Portugal)	(Pereira *et al*. 2006)
		1.56	Sado Estuary (Portugal)	(Lillebo *et al*. 2011)
	Carcinus sp.	0.50–0.83	River Aponwe (Nigeria)	(Falusi and Olanipekun 2007)
Fish	Not specified	0.20–0.98	East coast (USA)	(Mason and Lawrence 1999)
Amphibians	*Rana sphenocephala*	0.67–1.51	South Carolina (USA)	(Unrine and Jagoe 2004)
Terrestrial				
Funghi	*Cantharellus cibarius*	0.20–3.80	Poland	(Falandysz et al. 2012)
	Amanita rubescens	0.83–24.0	Poland	(Drewnowska et al. 2012)
	Xerocomus chrysenteron	25.7–33.9	Lugo (Spain)	(Melgar et al. 2009)
	Boletus pinophilus	324–491		
Plants	*Oriza sativa*	4.20	Guizhou (China)	(Meng et al. 2010)
	Rumex induratus	1231	Almadén (Spain)	(Moreno-Jiménez et al. 2006)
	Mimosa pudica	0.16–2.33	Sagua la Grande (Cuba)	(Gonzalez 1991)
	Marrubium vulgare	131		

a) corrected for soil organic matter content

Table 4.7 Biota-soil accumulation factors (BSAF) for mercury calculated as concentration in biota divided by concentration available in sediments (aquatic organisms) or soil (terrestrial organisms) (continued)

Organism	*Species*	BAF	Sampling area	Source
Terrestrial				
Earthworms	*Drawida sp.*	0.04 – 0.54	Huludao City (China)	(Zhang et al. 2009)
	Allolobophora sp.			
	Limnodrilus sp.			
	Eisenia fetida	0.60–3.10	Maryland (USA)	(Burton et al. 2006)

4.5.2 Diet-related effects in mercury bioaccumulation

Our data did not provide a clear relationship between diet and BSAF, most likely because of the combination of a great variability in BSAF (5.81–938) and a limited sample size. Because complex relationships require very large data sets to detect them, we cannot exclude that an increase in the number of sampled barn owl territories could have revealed a stronger relationship between diet and bioaccumulation. Several studies have shown that diet composition can influence concentration of contaminants in top predators (Lindberg and Odsjö 1983; Elliott *et al*. 1996; Anthony *et al*. 1999; Mañosa *et al*. 2003), which also specifically applies to Hg in raptors (Palma *et al*. 2005) and owls (Lourenço *et al*. 2011) from south Portugal.

The highest Hg concentrations were associated with Bonelli's eagles (*Hieraaetus fasciatus*) feeding on higher proportion of secondary consumers, whereas lower Hg concentrations were associated with diets mostly based on herbivores (Palma *et al*. 2005). Higher trophic level of prey also influenced Hg concentrations in the eagle owl (*Bubo bubo*); however, concentrations in primary consumers (herbivores) showed little variation and were not related with Hg concentrations in this predator (Lourenço *et al*. 2011). These studies suggest food web length may be a major source of variation in Hg contamination in terrestrial food webs, and therefore an effect of diet in BSAF should be expected.

In our study area diet diversity was similar between basins not showing any particular spatial variation patterns. Nevertheless, insectivore prey were more frequent in the Tagus basin, most likely resulting in a less contaminated area having BSAF values comparable to

those in a more contaminated area (Sado and Guadiana basins). The frequency of insectivores in barn owl diet seems to be lower in the Tagus basin compared with other areas in Portugal, where *Crocidura* spp. can be the main prey type (see Roque 2003 and references herein). This suggests diet-related effects in Hg concentrations in barn owl feathers could be determinant for spatial patterns in bioaccumulation, despite we could not find an overall relation with BSAFs variation. Wildlife exposure to metal contamination may vary considerably in species with an opportunistic feeding strategy as the barn owl (Taylor 1994; Roulin 2002), for instance due to spatial and temporal variation in the availability of different prey items (Schipper *et al*. 2012). Prey availability can in turn be influenced by the hunting habitats (Tomé *et al*. 2011). Moreover, seasonal variations may also occur: in the Tagus basin, diet diversity in April–August is higher in terms of prey frequency (1.04) but lower in terms of biomass (0.10), meaning that barn owls hunt more types of prey during the period they are feeding their nestlings, but apparently select prey items with similar body size (Roque 2003). These factors could probably also affect Hg bioaccumulation, introducing temporal variation. A possible underestimate of local and perhaps discontinuous peaks in metal contamination may also have resulted from aggregation of spatial and temporal variation (Schipper *et al*. 2012). In order to understand potential relationships between diet, Hg concentrations in the predator, and their effects in bioaccumulation, further studies should be conducted, including (1) a larger sample size, (2) spatio-temporal variation in barn owl diet, and (3) a quantification of Hg concentrations in prey.

4.5.3 Habitat-related effects in mercury bioaccumulation

Land uses that better explain variation in Hg concentrations in feathers and soil are permanently irrigated land (*e.g.* rice fields, corn fields) and heterogeneous agricultural areas (mosaic of small plots with annual crops, pasture and/or permanent crops), suggesting a relevant effect of irrigation and landscape structure in the processes affecting Hg distribution. Despite the similar relationship between both land uses and Hg in soil and feathers (increasing area occupied by each land use was associated with lower Hg concentrations), land uses do not affect BSAFs equally. Therefore, land use seems to influence the magnitude of bioaccumulation from Hg concentrations in soil to Hg concentrations in feathers. Permanently irrigated land did not produce an assessable outcome in BSAFs, suggesting that there is no obvious trend in Hg bioaccumulation from soil

to feathers in this land use. Although Hg concentrations in soil and feathers in permanently irrigated lands are lower than in other land uses, this apparently does not lead to Hg bioaccumulation below the average. The low contamination in permanently irrigated land is compatible, for instance, with this land use receiving lower load of Hg-containing agricultural inputs (including chemicals, bio-solids, manures and compost) and/or promoting a faster Hg elimination (*e.g.* by leaching, crop harvest, surface runoff and volatilization; Chang and Page 2000). This outcome could seem unanticipated, since irrigation is a possible source of contamination (Ackerman and Eagles-Smith 2010). Moreover, intermittent wetting and drying of wetland habitats – including rice fields – is frequently associated with increased MeHg production and bioaccumulation (Hall *et al*. 2008; Ackerman *et al*. 2010). On the contrary, irrigation may result in the elimination of elemental Hg, when below the water saturation of soil surface. This could be caused by competition of the more polar water molecule with Hg for binding sites, which are desorbed from soil particles into soil gas and dissolved in the soil water. Then, the process of evaporation facilitates movement of Hg to the soil surface where it is subsequently released (Gustin and Stamenkovic 2005). In agreement, studies with invertebrates and fish suggested that inorganic Hg became methylated, concentrated, and transported in the direction of water flow in wetlands (Ackerman *et al*. 2010; Ackerman and Eagles-Smith 2010). This metal has a strong tendency to adsorb to soil complexes, increasing the probability of off-site contamination due to transportation to aquatic systems in surface water runoff and between water bodies in suspended solids (Matthews *et al*. 1995). Therefore, it is possible that permanently irrigated areas may instead be increasing off-site Hg contamination. Areas with higher proportion of permanently irrigated land were also hypothesised to be related with lower contamination with organochlorine compounds in the barn owl, because of a faster decontamination related with higher variation in soil mobilization and humidity patterns (I. Roque, R. Lourenço, A. Marques, E. Martínez-López, S. Espín, S.A. García-Fernández, J. Rabaça, A. Roulin unpublished data). Regarding the effects of this land use in Hg concentration in feathers, there are evidences that bioaccumulation pathways in wetlands are complex, underlining the importance of using several taxa at different trophic levels to examine MeHg bioaccumulation (Ackerman *et al*. 2010; Ackerman and Eagles-Smith 2010). One effect could be, for instance, the different bioaccumulation in specific prey types that may be in the base

of the barn owl food web, since Hg concentrations of different invertebrates collected at the same locations and time periods may not be correlated (Ackerman *et al*. 2010).

On the other hand, heterogeneous agricultural areas seem to increase bioaccumulation in barn owls. These systems are characterized by small-scale land use heterogeneity, with a mix of small parcels with diverse annual crops, pasture and/or permanent crops, including areas mainly occupied by agriculture, interspersed with natural areas (Néry 2007). At a wider scale, vegetation patterns are known to influence Hg retention in soil due to the binding of Hg with the functional groups of soil organic matter (Eagles-Smith *et al*. 2016). Also Hg concentrations are largely influenced by plant productivity, driven by water availability (Obrist *et al*. 2016). Therefore, it is possible that similar processes influence Hg distribution at a smaller scale. Alike, we hypothesize that in a similar way that precipitation gradients and geomorphic variation create diverse landscapes with influence on terrestrial Hg pools (Eagles-Smith *et al*. 2016; Obrist *et al*. 2016), a similar smaller-scale process dependent on irrigation (as for rainfall deposition) and soil physical-chemical properties (as for geomorphic variation) could create small-scale patterns in soil Hg concentrations, with consequences in Hg bioaccumulation. How the resulting heterogeneous Hg distribution could influence BSAF is complex, since barn owls show lower Hg concentrations in territories with more heterogeneous agricultural areas but still there is an increasing effect in BSAF of this land use. This could possibly depend on the influence of small-scale landscape diversity in diet, influencing diet composition in barn owls by mediating prey abundance and availability. This suggests complex relations that are not explainable by univariate models. Further studies with larger sample size are needed in order to explore the effect of landscape diversity in Hg and relationships with bioaccumulation along the food web structure. The prospect of a complex pattern related with diet is also reinforced by the marginal influence of permanent crops in BSAF and in Hg concentrations in feathers, suggesting lower bioaccumulation in barn owl territories with a larger area with permanent cultures should be influenced by the food web (which in turn determines barn owl diet composition).

4.5.4 Effect of industrial emissions in mercury bioaccumulation

The absence of a relationship among industrial Hg emissions and BSAF and Hg concentrations in feathers is in agreement with the finding that biological mercury exposure is decoupled from inorganic mercury sources, highlighting the importance of local processes driving MeHg concentrations (Eagles-Smith *et al*. 2016), which is the key bioaccumulative form of Hg. Regarding Hg concentrations in soil, the absence of a pattern is possibly related with the sampling sites being too distant from the power plants to show a direct effect of their emissions. Enriched Hg contamination in soils has been reported within 15 km from coal-burning power plants (Rodríguez Martín and Nanos 2016). Our sampling sites have a mean minimum distance of 35.9 ± 26.0 km to the nearest power plant and only 8.7% are in the range of 15 km. Additionally, industrial sources of atmospheric Hg are also reported to cause increased concentrations in soils in the main wind direction (Biester *et al*. 2002). Higher soil contamination in the southernmost Sado-Guadiana basin is in accordance with an effect of atmospheric dispersion and long distance transport, since main wind direction is NW-S or NW-SE (Costa *et al*. 2006). Therefore, contamination in the area is likely to result from a cumulative effect of several industrial sources, which is agreeing with the absence of a clear spatial pattern. Most of our sampling sites are located in an wide area where airborne Hg concentrations – detected in lichens – range between 0.3 and 0.6 μg g^{-1} (Freitas 1999), also supporting a broad regional-scale impact of industrial emissions.

4.6 Conclusions

There is great variation in BSAFs in the barn owl, possibly linked with different agricultural land uses, apparently affected by factors influencing both Hg concentrations in soil and in feathers. Irrigated and heterogeneous agricultural areas, which are associated with lower soil and barn owl contamination, can possibly be used as proxy of lower Hg agricultural input and/or faster Hg decontamination (in case of permanently irrigated areas). Irrigated and heterogeneous agricultural areas, and permanent crops, associated with lower Hg contamination in feathers, should be further inspected for food web-related effects, because of potential variations in prey features: species-specific Hg bioaccumulation, diversity, abundance and/or accessibility. A specific land use may not affect BSAF and still produce effects in both concentrations in feathers and soil (i.e. show no obvious trend in Hg

bioaccumulation from soil to feathers). Therefore, as tools for interpreting the factors affecting bioaccumulation, BSAF should be analyzed together with Hg concentrations in biota and soil. Biota-soil accumulation factors in raptors seem to be useful indicators of contaminant dynamics in terrestrial systems, since they allow for raising specific hypothesis in terms of the environmental fate of contaminants like Hg.

5

Organochlorine pesticides in barn owl (*Tyto alba*) feathers and livers: matrix-related variation, spatial patterns and time trends

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5 Organochlorine pesticides in barn owl (*Tyto alba*) feathers and livers: matrix-related variation, spatial patterns and time trends

5.1 Abstract

The organochlorine compounds (OC) include the most prevalent synthetic pesticides that have been historically used in agriculture and which are still detected in the environment decades after their restriction. Wildlife biomonitoring studies may help evaluating organochlorine pesticides (OCPs) exposure and associated effects. Since the barn owl (*Tyto alba*) is a widespread raptor associated with farmland where OCPs may be particularly prevalent, we used this species as a case-study to evaluate feathers as a non--destructive biomonitoring tool translating OCPs in livers of raptors, and also to check possible relations with land uses. We measured the concentrations of 16 OCPs in 15 primary feathers and 15 livers from barn owl carcasses collected on roadsides in Tagus and Évora regions in south Portugal. Total OCPs mean concentration was 8 003 ng g^{-1} in feathers and 178 ng g^{-1} in livers. All compounds were detected in feathers while in livers δ-HCH, endosulfan sulphate, DDT and DDD were not detected. The high β-HCH and heptachlor concentrations found in feathers (4 587 and 2 530 ng g^{-1}, respectively) most likely derived from external contamination. The DDT metabolite DDE (45.4 ng g^{-1}) was the OCP with the highest concentration in livers. Possibly there has been continuous contamination by lindane over the last 30 years, and an accentuated decrease in heptachlor epoxide, DDT and aldrin. However, both matrices suggested an exposure to recently released heptachlor, which may have an industrial source. In general, our results suggest a similar temporal use and dosage between areas. However, the absence of some OCPs in Tagus which are present in Évora and a trend for lower OCPs concentrations in the former, suggest that the Tagus area is in a more advanced stage of decontamination. This may be due to a faster OCPs degradation facilitated by a more intensive fluctuation of soil humidity in the Tagus area. Our results suggest the barn owl may be a good biomonitor of environmental contamination with OCPs. Feathers may be particularly suitable as biomonitoring tools to detect legacy environmental contaminants, because these may not accumulate in liver while still present in the environment in low concentrations.

5.2 Introduction

Persistent organic pollutants (POPs) are ubiquitous chemicals, highly resistant to degradation and susceptible to bioaccumulation, therefore representing a potential health risk to humans and wildlife (UNEP 2011). A particular group of POPs, the organochlorine compounds (OCs), includes the most prevalent synthetic pesticides that have been broadly used in agriculture in the second half of the 20th century (Barr and Needham 2002; Barr 2008). These organochlorine pesticides (OCPs) can be classified in three groups: (1) dichlorodiphenyltrichloroethane (DDT) and related compounds, (2) cyclodiene insecticides (aldrin, dieldrin, endrin, heptachlor and endosulfan) and (3) isomers of hexachlorocyclohexane (HCH) (Mitra *et al*. 2011). The known impact of these substances on humans includes neurotoxic, endocrine disruptive and carcinogenic effects (Ritter *et al*. 1995; Jaga and Dharmani 2003; Wasi *et al*. 2013). Despite OCPs concentrations detected in wildlife are infrequently considered to be a direct cause of death, these are often reported as a cause of immunosuppression, hormone disruption and disorder of the nervous and reproductive systems (Denneman and Douben 1993; Furness *et al*. 1993; Martínez-López, 2005). Moreover, the accumulation of OCPs residues in body fat reserves as a result of a long term exposure to low concentrations, is also of concern: under stressful conditions (*e.g.* migration, food shortage, etc.) resulting in a rapid depletion of fat reserves, OCPs residues are released into the bloodstream and mobilized to different organs such as the brain, where they may attain toxic levels and cause acute poisoning (Friend and Franson 1999).

Given their hazardous effects, OCPs were interdicted in developed countries and replaced by less persistent pesticides. In Portugal the use of OCPs was regulated for the first time in 1988 (Decree-Law 347/88), after agreement between the government and the pesticide companies restricting the trade of dieldrin, heptachlor and DDT in 1974, and of aldrin, endrin, hexachlorobenzene and toxaphene in 1986 (APA 2010). In concurrence with the European Directive 79/117/CEE nine OCPs were restricted by decree, including DDT, the 'drins' (aldrin, dieldrin and endrin), heptachlor, and HCH (Ordinance 660/88). However, these substances are still detected decades after in the physical environment (Cerejeira *et al*. 2003; Villaverde *et al*. 2008; Cardoso *et al*. 2009; Carvalho *et al*. 2009), in food products (Correia-Sá *et al*. 2013; Blasco *et al*. 2004), in wildlife (Antunes-Maderia and Maderia 1989; Antunes and Gil 2004; Mathias *et al*. 2007; Van den Steen *et al*. 2009; Guimarães *et al*. 2010)

and in humans (Ferreira *et al*. 1990; Cruz *et al*. 2003; Lino and da Silveira 2006; Lopes *et al*. 2014).

Over the last 50 years, Portuguese agriculture has evolved from the free use of pesticides to the Integrated Pest Management (IPM), which became mandatory for professional agriculture with the latest reform of the Common Agricultural Policy (CAP) in 2014 (Decree-Law 256/2009). Accordingly, the sale of OCPs decreased 99.5% between 2002 and 2008 (Vieira 2004; DGADR 2009a; DGADR 2010a). Nevertheless, information related to the pesticides used in Portugal is not available, and assessment of environmental indicators to weigh up the effects of pesticides in the environment is lacking (Costa and Godinho 2012).

Wildlife biomonitoring studies are useful to assess spatial and temporal trends in concentrations of environmental chemicals and investigate related effects on populations: consequently, they can provide early warning of potential impacts in humans and protected wildlife species (van der Schalie *et al*. 1999; Burger and Gochfeld 2004; Reif 2011; Gómez-Ramírez *et al*. 2014; Espín *et al*. 2016). Birds of prey are susceptible to contaminant bioaccumulation and biomagnification due to their position at the top of food chains, often with complex trophic links connecting aquatic and terrestrial ecosystems (van Drooge *et al*. 2008; Mateo *et al*. 2012). As a result, measurable OCPs concentrations in tissue residues can be used to evaluate exposure and effects (Martínez-López *et al*. 2009; Gómez-Ramírez *et al*. 2012; Gómez-Ramírez *et al*. 2014; Espín *et al*. 2016). A widespread and common resident species, the barn owl (*Tyto alba*), can often be associated with man-made structures and is known for its fidelity to nest sites (Taylor 1994, BOT 2012, Dreiss and Roulin 2014), allowing for monitoring the same territories for long time periods using minimally invasive methods (*e.g.* feathers, blood). Moreover, a great number of carcasses can be collected on road sides (Massemin and Zorn 1998; Silva *et al*. 2008), also allowing for access to internal tissues that otherwise would be unattainable for ethical and legal reasons. The barn owl therefore meets the characteristics for a sentinel species, as required by the National Research Council (1991).

As an opportunistic meso-predator that hunts in open farmland (Bunn *et al*. 1982; Roulin 2002), the species is most likely associated with contamination from agricultural source, which includes OCPs. Concentrations of these contaminants in feathers and internal tissues

can be affected by several factors (see García-Fernández *et al*. 2013) and there are ambiguous evidences in literature documenting strong (Jaspers *et al*. 2007a; Eulaers *et al*. 2011; Jaspers *et al*. 2011; Rajaei *et al*. 2011) and low significant correlations (Dauwe *et al*. 2005; Jaspers *et al*. 2007b; Jaspers *et al*. 2009; Espín *et al*. 2010a; Espín *et al*. 2014) between OCPs levels in feathers and internal tissues. OCPs bind to keratin structure during the feather growth period, after which vascular connections undergo atrophy and compound concentrations remain stable (García-Fernández *et al*. 2013). Since feathers reflect blood concentrations at the time of feather formation, the time elapsed until sampling should be considered when interpreting concentrations, particularly in comparisons with internal tissues (Espín *et al*. 2012; García-Fernández *et al*. 2013). Given the lipophilic nature of OCPs, the highest internal concentrations of these contaminants are expected to be found in adipose tissue, followed by liver and muscle (García-Fernández *et al*. 2013). For this reason, concentrations are often higher in internal tissues than in feathers (Espín *et al*. 2010a; Jaspers *et al*. 2011; Rajaei *et al*. 2011). However, less persistent compounds that are more easily metabolized may be found at lower concentrations in fat or liver and in higher levels in the bloodstream for a limited time, due to lipid mobilization (García-Fernández *et al*. 2013). If this temporary shift occurs during feather formation, feathers can show proportionally higher concentrations than internal tissues (Dauwe *et al*. 2005; Jaspers *et al*. 2007b; Rajaei *et al*. 2011). Additionally, concentrations in feathers may also be affected by external contamination, which is a topic in need of further research (Espin *et al*. 2014).

Considering the easy access to barn owl feather and internal tissue samples, the lack of clear information in the literature on the relationship between contaminant concentrations in those matrices, and the diversity of agricultural land uses in central and south Portugal, we conducted a study aiming to: (1) evaluate feathers as a non-destructive biomonitoring tool comparing OCPs concentrations with those measured in livers; (2) check differences in OCPs accordingly in two distinct regions in Portugal; and (3) evaluate the suitability of the barn owl, a widespread raptor associated with agricultural uses, as a biomonitor for OCPs.

5.3 Methods

5.3.1 Study areas

Samples were collected along roads in two areas in central Portugal with different farmland uses: lower Tagus River (hereafter Tagus; 38°56'N–8°55'W) and Évora (38°33'N–7°54'W), with a mean distance of 82 km. The climate is Mediterranean, with rains concentrated in winter, and characterized by hot dry summers and mild winters. Landscape is mostly plain or undulating. The Tagus area encloses the left margin of the Tagus River in the vicinity of its estuary, located in the metropolitan area of Lisbon. Based on the land uses of CORINE Land Cover 2006 (European Environment Agency 2007), farmland and forest uses roughly allocate half of the area each, both in Tagus and Évora (farmland: 47% in Tagus and 46% in Évora; forest: 43% in Tagus and 40% in Évora). In Tagus, dominant land use is production forests (32%), followed by complex cultivation patterns (15%) and irrigated lands, including rice fields (13%). Vineyards and olive groves occupy ca. 12% of the area. In Évora, dominant land use is non-irrigated arable land (34%) followed by agro-forestry areas (29%). Here, production forests also occupy ca. 18% of the area.

5.3.2 Collection of samples

Barn owl feathers and livers were collected from 15 road-killed birds found on roadsides between 2009 and 2012. Six individuals were found in the Tagus area and nine in Évora. According with plumage moult (Martínez *et al*. 2002) sample owls were one (n = 2), two (n = 6) and three years old (n = 3). A primary feather was randomly plucked from each bird, resulting in 15 samples with different positions in the wing. Liver was excised from each owl. Feather and liver samples were stored in individual transparent plastic bags and in aluminium foil, respectively, and kept frozen at -20°C until analysis.

5.3.4 Organochlorine analysis

Primary feather and liver samples were analysed for 16 OCPs: four HCH isomers (α--HCH, β-HCH, γ-HCH – or lindane – and δ-HCH), three endosulfan related compounds (endosulfan I, endosulfan II and endosulfan sulphate), four 'drins' (aldrin, dieldrin, endrin and endrin aldehyde), three DDT related compounds (p,p'-DDT, p,p'-DDD and p,p'-DDE) and

two heptachlor related compounds (heptachlor and heptachlor-epoxide). The analytical procedures were based on the method described by Espín *et al*. (2010b; 2012) for feathers and Espín *et al*. (2010a) for liver samples. In order to remove external contamination from the feather surface, prior to the analytical determination, a brief washing process was performed with tap water, distilled water and Milli-Q water, and two pairs of tweezers were used to separate the barbs of the vane. Identification and quantification was based on an external standard (EPA Pesticide Mix 48858, Supelco, USA), and methoxychlor (1 mg mL^{-1}) supplied by PolyScience® was used as an internal standard. In order to compare results and check the repeatability in the chromatograms a volume of 10 µL of methoxychlor was added to samples and standards. Spiked samples mean recoveries ranged from 46% to 146% depending on the compound and matrix. Detection limits ranged from 0.03 to 0.54 ng g^{-1}. OCPc concentrations were expressed as ng g^{-1}.

5.3.5 Statistical analysis

Reported OCPs values represent the mean concentration ± standard deviation, median and range, and frequency of detection. Total concentrations of OCPs groups (ΣOCP) were calculated as the sum of individual compound concentrations: DDT and metabolites (ΣDDT) corresponded to the sum of p,p´-DDE, p,p´-DDD and p,p´-DDT; hexaclorocyclohexanes (ΣHCH) incorporated α, β, δ and γ-isomers; heptachlor group (ΣHeptachlor) included heptachlor and its epoxide; ΣDrins represented the sum of endrin, aldrin and dieldrin; and ΣEndosulfan included endosulfan I and II. The percentage of individual compound concentrations in their group was also calculated for feathers and livers.

Since our data were not normally distributed even following several transformation trials, the non-parametric paired samples Wilcoxon test was used in order to detect differences between sampling matrices and Spearman correlations between feather and liver concentrations were also performed. The Mann–Whitney test was used in order to detect differences between areas in both feather and liver concentrations. The level of significance (two tailed) for these tests was set at $p<0.05$. All statistical analyses were conducted using R software 3.1.1 (R Core Team 2014).

5.4 Results

5.4.1 General outline in organochlorine pesticides concentrations in barn owls

All the monitored OCPs were detected in barn owl samples: the 16 compounds were detected in feathers while in livers four OCPs were not detected (δ-HCH, endosulfan sulphate, DDT and DDD, Table 5.1). Total OCPs mean concentration was 8 120 ± 6 432 ng g^{-1} in feathers and 178 ± 112 ng g^{-1} in livers (Table 5.1). ΣHCH and ΣHeptachlor were measured in feathers in particularly high concentrations when compared to the other OCP groups (5 274 ± 4 484 ng g^{-1} and 2 555 ± 2 439 ng g^{-1}, respectively, Table 5.1), representing together 96% of the ΣOCP in feathers. This difference was not observed in livers, in which ΣHCH (30%), ΣDrins (26%) and ΣDDT (26%) represented a similar burden (Table 5.1).

First year barn owls showed significantly higher mean concentrations of heptachlor epoxide than second year birds both in livers (1.22 ± 1.16 ng g^{-1} and 0.093 ± 0.246 ng g^{-1}, respectively; w = 36; p = 0.024; N = 15) and un-moulted feathers (40.1 ± 39.0 ng $g^{-1}$and 9.13 ± 9.81 ng g^{-1}, respectively; w = 36; p = 0.037; N = 13 – two moulted feathers were excluded from this test).

Table 5.1 Concentrations of organochlorine pesticides (ng g^{-1}) in livers (wet weight) and feathers (dry weight) of barn owls from Portugal in 2009-2012. Values are presented as mean ± standard deviation, median and range, and frequency of detection (%). Paired sample Wilcoxon test results on differences in concentration of organochlorine pesticides between matrices and Spearman correlations are also presented

Organochlorine	Feathers (n=15)	Livers (n=15)	Wilcoxon	Spearman
α-HCH	121 ± 95.1	3.90 ± 7.48	v = 0	ρ = -0.084
	81.7 [29.3–398]	1.37 [nd–30.1]	$p < 0.001$	$p = 0.766$
	100	87		
β-HCH	4 587 ± 4 375	15.3 ± 33.0	v = 0	ρ = 0.073
	2 882 [1 076–18 693]	nd [nd–109]	$p < 0.001$	$p = 0.795$
	100	27		

Table 5.1 Concentrations of organochlorine pesticides (ng g^{-1}) in livers (wet weight) and feathers (dry weight) of barn owls from Portugal in 2009-2012. Values are presented as mean ± standard deviation, median and range, and frequency of detection (%). Paired sample Wilcoxon test results on differences in concentration of organochlorine pesticides between matrices and Spearman correlations are also presented (continued I)

Organochlorine	Feathers (n=15)	Livers (n=15)	Wilcoxon	Spearman
δ-HCH	501 ± 423 339 [76.8–1679] 100	nd	v = 0 $p < 0.001$	-
Lindane	64.2 ± 58.7 37.0 [8.34–222] 100	33.3 ± 56.5 9.45 [3.65–230] 100	v = 33 $p = 0.135$	$\rho = -0.018$ $p = 0.954$
Heptachlor	2 531 ± 2 417 1 268 [177–7 854] 100	20.2 ± 29.3 4.41 [nd–75.0] 80	v = 0 $p < 0.001$	$\rho = 0.272$ $p = 0.326$
Heptachlor epoxide	24.2 ± 29.5 11.8 [nd–93.0] 73	0.61 ± 0.89 nd [nd–2.98] 47	v = 0 $p = 0.004$	$\rho = 0.719$ $p = 0.002$
Aldrin	28.2 ± 46.4 11.6 [nd–189] 67	0.12 ± 0.46 nd [nd–1.86] 7	v = 0 $p = 0.006$	$\rho = 0.441$ $p = 0.100$
Dieldrin	16.1 ± 28.0 4.70 [nd–93.9] 60	16.9 ± 46.6 2.88 [nd–189] 67	v = 39 $p = 0.414$	$\rho = -0.015$ $p = 0.978$
Endrin	128 ± 475 nd [nd–1 907] 13	12.8 ± 35.2 nd [nd–137] 33	v = 17 $p = 0.673$	$\rho = -0.270$ $p = 0.331$
Endrin aldehyde	15.6 ± 58.4 nd [nd–234] 7	16.6 ± 33.5 nd [nd–116] 27	v = 10 $p = 0.590$	$\rho = -0.159$ $p = 0.572$

Table 5.1 Concentrations of organochlorine pesticides (ng g^{-1}) in livers (wet weight) and feathers (dry weight) of barn owls from Portugal in 2009-2012. Values are presented as mean ± standard deviation, median and range, and frequency of detection (%). Paired sample Wilcoxon test results on differences in concentration of organochlorine pesticides between matrices and Spearman correlations are also presented (continued II)

Organochlorine	Feathers (n=15)	Livers (n=15)	Wilcoxon	Spearman
Endosulfan I	10.5 ± 26.8 nd [nd–82.2] 13	12.3 ± 19.2 3.78 [nd–64.4] 53	v = 29 *p* = 0.477	ρ = 0.076 *p* = 0.787
Endosulfan II	32.3 ± 58.8 nd [nd–220] 47	0.53 ± 1.07 nd [nd–3.99] 27	v = 3 *p* = 0.024	ρ = -0.067 *p* = 0.812
Endosulfan sulphate	7.45 ± 10.8 nd [nd–29.9] 40	nd	v = 0 *p* = 0.036	-
p.p'-DDT	45.9 ± 66.9 nd [nd–231] 47	nd	v = 0 *p* = 0.022	-
p.p'-DDD	0.78 nd [nd–11.7] 7	nd	v = 0 *p* = 1	-
p.p'-DDE	6.43 ± 16.1 nd [nd–64.1] 33	45.4 ± 48.7 25.4 [1.93–162] 100	v = 107 *p* = 0.005	ρ = 0 *p* = 1
Σ HCH	5 274 ± 4 484 3 476 [1 219–19 430] 100	52.5 ± 59.2 31.2 [6.30–231] 100	v = 0 *p* < 0.001	ρ = -0.107 *p* = 0.705

Table 5.1 Concentrations of organochlorine pesticides (ng g^{-1}) in livers (wet weight) and feathers (dry weight) of barn owls from Portugal in 2009-2012. Values are presented as mean ± standard deviation, median and range, and frequency of detection (%). Paired sample Wilcoxon test results on differences in concentration of organochlorine pesticides between matrices and Spearman correlations are also presented (continued III)

Organochlorine	Feathers (n=15)	Livers (n=15)	Wilcoxon	Spearman
Σ DDT	53.2 ± 73.3 8.26 [nd–247] 67	45.4 ± 48.7 25.4 [1.93–162] 100	v = 56 *p* = 0.85	ρ = 0.011 *p* = 0.969
Σ Drins	188±490 22.6 [nd–2 001] 87	46.4±82.6 10.1 [nd–328] 80	v = 47 *p* = 0.49	Rho=-0.118 P=0.674
Σ Endosulfan	50.3 ± 85. 23.0 [nd–324.9] 53	1 289 ± 20.0 3.78 [nd–68.4] 53	v = 16 *p* = 0.14	ρ = -0.111 *p* = 0.693
Σ Heptachlor	2 555±2 525 1 268 [189–7 940] 100	20.8±29.0 4.65 [nd–75.0] 93	v = 0 *p* < 0.001	ρ = 0.371 *p* = 0.174
Σ OCPs	8 120 ± 6 432 5 508 [1 605– 27 424] 100	178 ± 112 192 [22.0–448] 100	v = 0 *p* < 0.001	ρ = -0.179 *p* = 0.524

5.4.2 Differences in organochlorine pesticides contamination between barn owl feathers and livers

The compounds with the highest mean concentration in feathers were β-HCH (4 587 ± 4 375 ng g^{-1}) and heptachlor (2 531 ± 2 417 ng g^{-1}; Fig. 5.1 A, Table 5.1), while DDE (45.4 ± 48.7 ng g^{-1}) was the compound with the highest mean concentration in liver (Fig. 5.1 B, Table 5.1). The four HCH isomers and heptachlor were the most frequently detected compounds in feather samples (all individuals), and lindane (*i.e.* γ-HCH), α-HCH, DDE and heptachlor were

the most frequently detected in liver samples (80–100%, Table 5.1). The least prevalent family of compounds was endosulfan, detected in ca. half of the samples, both in livers and feathers (Table 5.1). Mean ΣOCP concentration was 46 times superior in feathers than in liver and median ΣOCP concentration was 29 times higher in feathers than in livers. Nevertheless, the concentrations of six compounds were similar in both matrices: lindane, dieldrin, endrin, endrin aldehide, endosulfan I and DDD (Table 5.1). Significant positive correlation between feather and liver concentrations were found only for heptachlor epoxide (ρ = 0.719; p = 0.002; Table 5.1). While β-HCH represents the highest percentage among the ΣHCH in feathers (87%), lindane is the isomer most accumulated in liver (64%; Fig. 5.2). Heptachlor represents 97–99% of its family in feathers and livers, while its epoxide represents only 1–3% (Fig. 5.2). Among ΣDrins, endrin is the most represented in feathers (68%), followed by aldrin (15%), while in livers dieldrin (37%) and endrin aldehide (36%) prevail over endrin (28%; Fig. 5.2). Endosulfan II is the most abundant compound of its family in feathers (64%) while in livers endosulfan I (96%) is the most represented (Fig. 5.2). Among ΣDDT, DDT has the highest percentage in feathers (86%) while DDE prevails in liver (100%; Fig. 5.2).

5.4.3 Organochlorine pesticides contamination in the two study areas

The compounds with the greatest mean concentration in feathers were the same in Évora and Tagus: β-HCH (3 873 ± 2 940 ng g^{-1} and 5 658 ± 6 426 ng g^{-1}, respectively; Table 5.2) and heptachlor (2 831 ± 2 383 ng g^{-1} and 2 081 ± 2 835 ng g^{-1}, respectively). In livers, the compound with the greatest mean concentration in both areas was DDE (49.3 ± 55.6 ng g^{-1} and 39.5 ± 45.8 ng g^{-1}, respectively in Évora and Tagus). The compound with the second greatest concentration differed between areas: lindane in Évora (47.9 ± 72.7 ng g^{-1}) and endrin aldehyde in Tagus (29.3 ± 45.6 ng g^{-1}). Five compounds were found in samples from Évora but not in Tagus: four compounds in feathers (endosulfan I, endrin and endrin aldehyde and DDD), and one in livers (aldrin) (Table 5.2). The four HCH isomers and heptachlor were the most frequently detected compounds in feather samples from both areas (in all individuals, except α-HCH; Table 5.2). Lindane was detected in all liver samples from both areas, but in Tagus dieldrin had also 100% frequency of detection while in Évora its prevalence was 44% (Table 5.2). There was a general trend for mean OCPs concentrations to be higher in Évora, except for β-HCH in feathers and livers, and heptachlor epoxide and

endrin aldehyde in livers. However, none of the isolated OCP mean concentrations differed significantly between study areas, ΣDrins in feathers was significantly higher in Évora than in Tagus (Table 5.2).

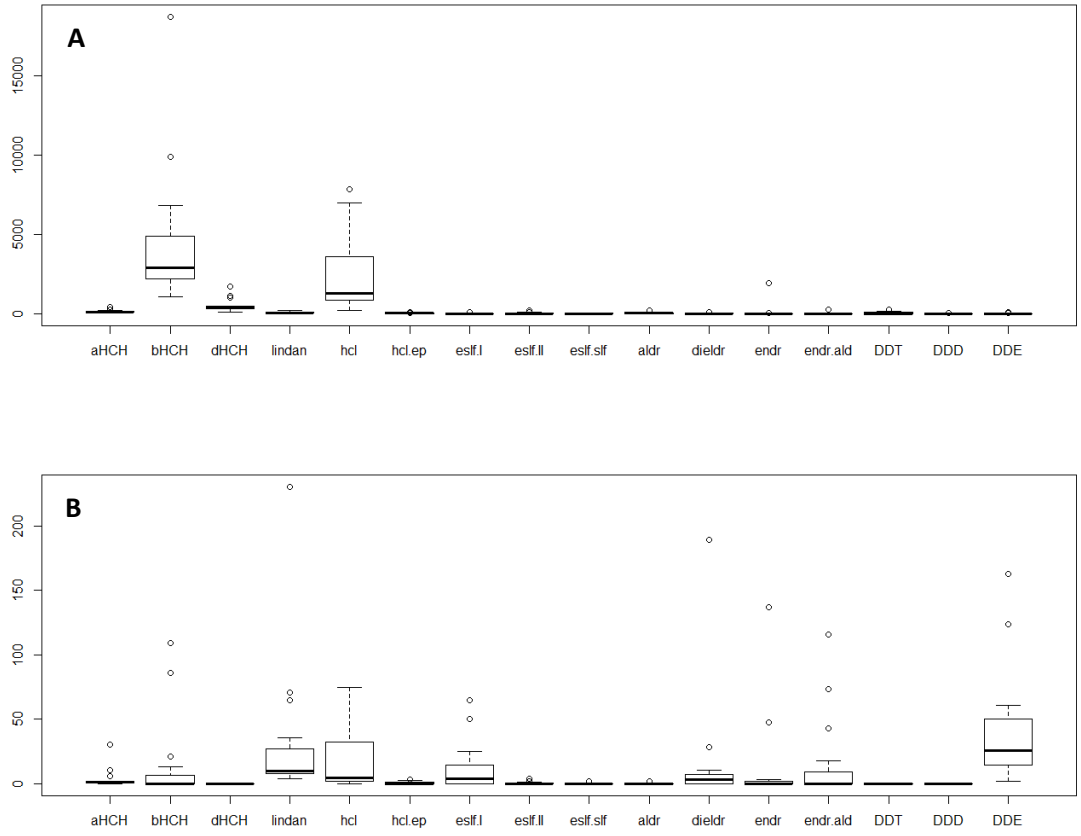

Figure 5.1 Concentration (ng g^{-1}) of 15 organochlorine pesticides (OCPs) in barn owl primary feathers (A) and livers (B). Box and whisker plots show the median, 25% quartiles and range

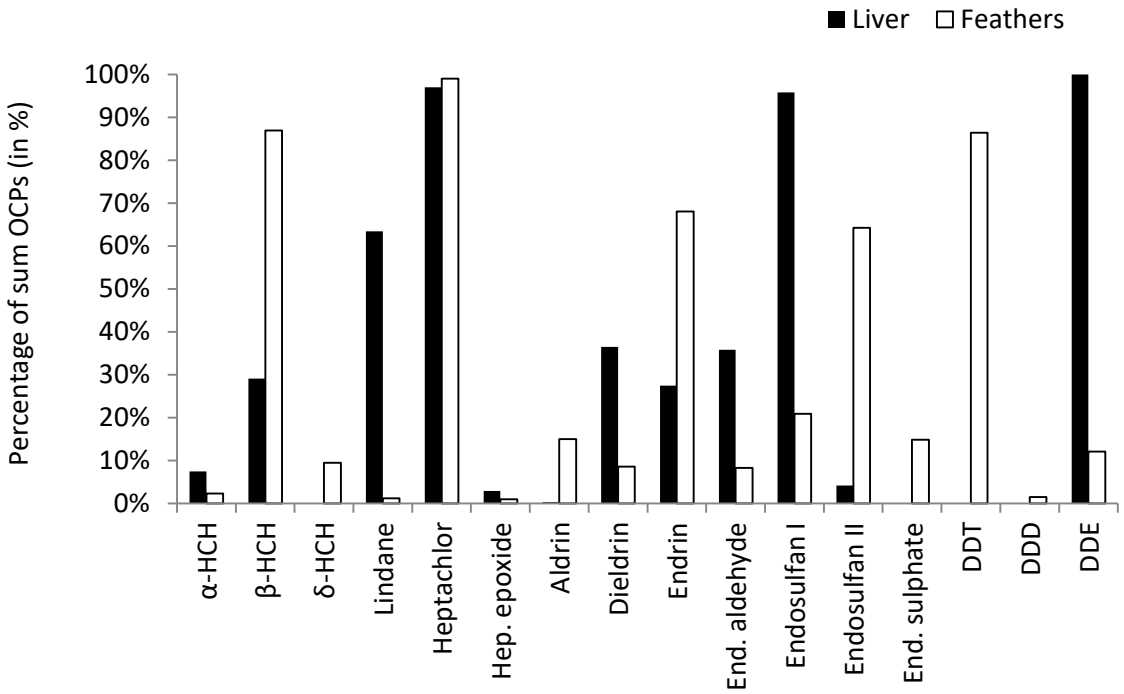

Figure 5.2 Relative concentrations of 15 organochlorine pesticides (OCPs) expressed as percentage of total concentrations of OCPs groups (ΣHCH, ΣHeptachlor, ΣDrins, ΣEndosulfan and ΣDDT) in barn owl primary feathers and livers from Portugal in 2009–2012

Table 5.2 Concentrations of organochlorine pesticides (ng g^{-1}) in feathers (dry weight) and livers (wet weight) of barn owls from Évora and Tagus (Portugal) in 2009-2012. Values are presented as mean ± standard deviation, median, range and frequency of detection (%). Mann-Whitney test results on differences in concentrations of organochlorine pesticides between areas are also presented

Organochlorine	Feathers (n = 15)		Livers (n = 15)	
	Évora (n = 9)	Tejo (n = 6)	Évora (n = 9)	Tejo (n = 6)
α-HCH	124 ± 114	117 ± 78.7	6.02 ± 9.60	0.72 ± 0.60
	72.3 [29.3–398]	91.6 [56.0–273]	1.81 [nd–30.1]	0.76 [nd–1.49]
	100	100	89	83
	w = 24; *p* = 0.78		w = 43.5; *p* = 0.06	
β-HCH	3 873 ± 2 940	5 658 ± 6 426	14.4 ± 36.1	16.6 ± 34.5
	2 838 [1 076–9 877]	2 997 [2 265–18 693]	nd [nd–109]	nd [nd–86.2]
	100	100	22	33
	w = 21; *p* = 0.53		w = 25; *p* = 0.82	

Table 5.2 Concentrations of organochlorine pesticides (ng g^{-1}) in feathers (dry weight) and livers (wet weight) of barn owls from Évora and Tagus (Portugal) in 2009-2012. Values are presented as mean ± standard deviation, median, range and frequency of detection (%). Mann-Whitney test results on differences in concentrations of organochlorine pesticides between areas are also presented (continued I)

Organochlorine	Feathers (n = 15)		Livers (n = 15)	
	Évora (n = 9)	Tejo (n = 6)	Évora (n = 9)	Tejo (n = 6)
δ-HCH	620 ± 531	324 ± 148	nd	nd
	482 [96.0–1 679]	333 [76.8–483]	nd	nd
	100	100	0	0
	w = 35; *p* = 0.39		-	
Lindane	69.3 ± 66.9	56.7 ± 55.4	47.9 ± 72.7	11.5 ± 12.1
	44.0 [8.34–222]	27.1 [13.7–130]	11.14 [7.31–230]	7.56 [3.65–35.7]
	100	100	100	100
	w = 31; *p* = 0.69		w = 43; *p* = 0.07	
Heptachlor	2 830 ± 2 383	2 081 ± 2 835	25.8 ± 34.7	11.9 ± 22.9
	1 743 [177–6 963]	1 008 [720–7 854]	4.65 [nd–75.0]	3.04 [nd–58.4]
	100	100	78	83
	w = 37 *p* = 0.27		w = 34; *p* =0.44	
Heptachlor epoxide	25.8 ± 31.1	21.9 ± 32.5	0.57 ± 0.80	0.67 ± 1.16
	11.8 [nd–93.0]	11.9 [nd–86.0]	nd [nd–2.12]	0.20 [nd–2.98]
	78	67	44	50
	w = 29; *p* = 0.86		w = 26.5 *p* = 1.0	
Aldrin	39.3 ± 58.6	11.6 ± 20.6	0.21 ± 0.62	nd
	18.3 [nd–189]	3.28 [nd–52.6]	nd [nd–1.86]	nd
	78	50	11	0
	w = 30; *p* = 0.17		w = 30; *p* = 0.50	
Dieldrin	23.5 ± 35.7	5.03 ± 8.03	22.6 ± 62.6	8.41 ± 10.1
	7.12 [nd–93.9]	nd [nd–18.2]	nd [nd–189]	3.87 [1.66–28.1]
	78	33	44	100
	w = 37; *p* = 0.25		w = 14; *p* = 0.13	
Endrin	213 ± 635	nd	20.8 ± 46.3	0.63 ± 1.02
	nd [nd–1 907]	nd	nd [nd–137]	nd [nd–2.32]
	22	0	33	33
	w = 32; *p* = 0.27		w = 30; *p* = 0.73	

Table 5.2 Concentrations of organochlorine pesticides (ng g^{-1}) in feathers (dry weight) and livers (wet weight) of barn owls from Évora and Tagus (Portugal) in 2009-2012. Values are presented as mean ± standard deviation, median, range and frequency of detection (%). Mann-Whitney test results on differences in concentrations of organochlorine pesticides between areas are also presented (continued II)

Organochlorine	Feathers (n = 15)		Livers (n = 15)	
	Évora (n = 9)	Tejo (n = 6)	Évora (n = 9)	Tejo (n = 6)
Endrin aldehyde	26.0 ± 78.0	nd	8.16 ± 24.5	29.3 ± 45.6
	nd [nd–234]	nd	nd [nd–73.4]	8.73 [nd–116]
	11	0	11	50
	w = 30; *p* = 0.50		w = 17; *p* = 0.15	
Endosulfan I	17.5 ± 34.8	nd	16.1 ± 25.0	6.54 ± 6.78
	nd [nd–82.2]	nd	nd [nd–64.4]	5.40 [nd–17.3]
	22	0	44	67
	w = 33; *p* = 0.27		w = 26; *p* = 0.95	
Endosulfan II	34.7 ± 73.7	28.7 ± 40.7	0.62 ± 1.37	0.39 ± 0.64
	nd [nd–220]	7.74 [nd–97.0]	nd [nd–3.99]	nd [nd–1.50]
	44	50	22	33
	w = 24.5; *p* = 0.80		w = 26; *p* = 0.94	
Endosulfan sulphate	10.5 ± 13.3	2.89 ± 5.18	nd	nd
	nd [nd–29.9]	nd [nd–12.8]	nd	nd
	44	50	0	0
	w = 34; *p* = 0.39		w = 26; *p* = 0.94	
p.p'-DDT	71.7 ± 79.9	7.26 ± 14.1	nd	nd
	81.5 [nd–231]	nd [nd–35.3]	nd	nd
	56	33	0	0
	w = 38; *p* = 0.18		-	
p.p'-DDD	1.30 ± 3.91	nd	nd	nd
	nd [nd–11.7]	nd	nd	nd
	11	0	0	0
	w = 30 *p* = 0.50		-	
p.p'-DDE	10.2 ± 21.0	0.79 ± 1.94	49.3 ± 55.6	39.53 ± 45.78
	nd [nd – 64.1]	nd [nd–4.74]	34.1 [1.93–162]	19.71 [2.615–123.6]
	44	17	100	100
	w = 35.5; *p* = 0.26		w = 3; *p* = 0.69	

Table 5.2 Concentrations of organochlorine pesticides (ng g^{-1}) in feathers (dry weight) and livers (wet weight) of barn owls from Évora and Tagus (Portugal) in 2009-2012. Values are presented as mean ± standard deviation, median, range and frequency of detection (%). Mann-Whitney test results on differences in concentrations of organochlorine pesticides between areas are also presented (continued III)

Organochlorine	Feathers (n = 15)		Livers (n = 15)	
	Évora (n = 9)	Tejo (n = 6)	Évora (n = 9)	Tejo (n = 6)
Σ HCH	4 685 ± 3 160 3 022 [1 219–10 808] 100	6 156 ± 6 541 3 571 [2 600–19 430] 100	68.3 ± 71.4 39.4 [8.69–231] 100	28.8 ± 35.2 13.4 [6.30–97.1] 100
	w = 23.5; *p* = 0.69		w = 40.5; *p* = 0.14	
Σ DDT	83.2 ± 86.1 81.5 [nd–247] 78	8.05 ± 13.8 2.37 [nd–35.3] 50	49.3 ± 55.6 34.1 [1.93–162] 100	39.5 ± 45.8 19.7[2.62–124] 100
	w = 41; *p* = 0.11		w = 31; *p* = 0.96	
Σ Drins	302 ± 643 52.7 [11.6–2 001] 100	16.7 ± 24.5 8.55 [nd–64.6] 67	51.8 ± 108 2.84 [nd–328] 67	38.4 ± 42.4 25.1 [3.14–119] 100
	w = 47; *p* = 0.02*		w = 16; *p* = 0.21	
Σ Endosulfan	62.7 ± 106 23.0 [nd–325] 56	31.6 ± 40.9 14.1 [nd–97.0] 50	16.7 ± 26.0 nd [nd–68.4] 44	6.93 ± 6.95 6.15 [nd–17.3] 67
	w = 29; *p* = 0.85		w = 26; *p* = 0.95	
Σ Heptachlor	2 857 ± 2 401 1 751 [189–7 056] 100	2 103 ± 2 866 1 015 [720–7 940] 100	26.3 ± 34.2 4.65 [nd–75.0] 89	12.6 ± 22.6 3.72 [0.65–58.4] 100
	w = 37; *p* = 0.27		w = 32; *p* = 0.61	
Σ OCPs	7 990 ± 4 743 6 272 [1 605–15 541] 100	8 315 ± 9 382 4 609 [3 858–27 424] 100	212 ± 121 209 [22.0–448] 100	126 ± 95.0 125 [23.3–246] 100
	w = 34; *p* = 0.46		w = 39; *p* = 0.18	

5.5 Discussion

5.5.1 Organochlorine pesticides in feathers and livers of Portuguese barn owls

Two contaminants are of special concern in our study: β-HCH and heptachlor (4 587 ng g^{-1} and 2 530 ng g^{-1}, respectively, in feathers). Their concentrations were approximately twofold the maximum concentrations reported to date in raptor feathers (2 250 ng g^{-1} and 1 450 ng g^{-1}, respectively, in Argentinean scavengers; Martínez-López *et al*. 2015). When compared with other European raptors, our results suggest a very high exposure of Portuguese barn owls to ΣHCH (5 274 ng g^{-1}) and heptachlor: mean concentrations in feathers were 19.8 and 9.6 times higher than the maximum value reported to date in this continent, respectively (ΣHCH_{max} = 266 ng g^{-1} in western marsh harrier (*Circus aeruginosus*) feathers from Greece; $heptachlor_{max}$ = 263 ng g^{-1} in European honey buzzard (*Pernis apivorus*) from Spain; van Drooge *et al*. 2008; Hela *et al*. 2006). Comparisons between HCH and heptachlor concentrations in feathers and liver are scarce. In Spanish razorbills (*Alca torda*) the ratio feather:liver of mean ΣHCH and ΣHeptachlor concentrations was approximately two (Espín *et al*. 2010, 2012). However, in Portuguese barn owl the ratio is 100 for ΣHCH and 123 for ΣHeptachlor, suggesting a possible effect of external contamination on feathers by these compounds. Differences in exposure and accumulation between barn owl and razorbill are possible due to differences in their ecology (*e.g.* diet, migratory behaviour, habitat, moult pattern; García-Fernández *et al*. 2013) and/or metabolization capacity (Dybing *et al*. 2002). However, we considered this extreme variation as an argument for further exploration on the possible effect of external contamination on the concentration measured in feathers. Unwashed feathers may show higher concentrations of certain compounds (*i.e.* lindane, heptachlor epoxide, DDE, endrin and endrin aldehyde) than washed feathers, suggesting that external contamination may affect the OCPs levels found in feathers (Espín *et al*. 2010). The washing techniques tested to date may not be effective in removing all external contamination by organic compounds (Espín *et al*. 2016).

Interpreting feather concentrations is complex because of the potential effect of external contamination, which is closely related with the moult strategy. Barn owls have a complex moult, replacing only 1-2 flight feathers in some years (Martínez *et al*. 2002; Hardey

et al. 2006). This protracted moult increases inter-feather variability because of age differences and its associated external contamination (Espín *et al*. 2016). All but two of the feathers we analyzed were first grown primaries, thus indicating contamination levels of individuals as nestlings, in the natal territory. Accordingly, feather samples in our study reflect mostly the exposure during growth as fledglings (1–2 months old), plus the potential external contamination. In contrast, liver concentrations reflect recent dietary exposure (Espín *et al*., 2016), in this case at the moment of death (3–36 months old). Therefore, the interval between feather growth (and associated deposition of pollutant in the feather) and collection of liver samples may sometimes be considerably large. During this period, post feather-growth changes in diet, spatial displacements (from nest sites to dispersal areas), and/or fat mobilization may alter liver OCPs concentrations. This may explain why we mostly found no correlation between concentrations in feathers and livers (a single positive significant correlation was found between feathers and livers for heptachlor epoxide). Therefore, the time elapsed between feather growth and the date of internal tissue sampling, seem to affect the associations between OCPs concentrations in feathers and internal tissues.

External contamination, which interferes with feather–liver correlations, has two main sources: (1) it can result from atmospheric deposition (*i.e.* exogenous) on feathers; and (2) it can have origin in preen gland oil (*i.e.* endogenous), which birds spread on feathers. In this last case it may improve correlations, since a part of the external contamination depends on internal concentrations (Jaspers *et al*. 2008; García-Fernández *et al*. 2013). The positive significant correlation we found in heptachlor epoxide concentrations between feathers and livers, may suggest no effect of external contamination in this OCP. Still, this correlation may be just a consequence of a parallel negative trend with age in feather and liver OCPs concentrations, rather than having a direct relation between matrices. In addition, as heptachlor epoxide is the main metabolite of heptachlor and is more stable than the parent compound (Xiao *et al*. 2011; Purnomo *et al*. 2013), it may not decrease with age.

At least two factors may reduce OCPs concentrations along time, thus explaining differences between barn owl un-moulted feathers. The first is mechanical abrasion and washing, which may alter feather structure (*e.g.* reduction in feather surface and pigment fade; Figuerola and Senar 2005; Surmacki *et al*. 2011; Flinks and Salewski 2012), and

consequently cause a noticeable decrease in OCPs concentration. The second explanation is a depletion of surface OCPs with bathing. Despite bathing is rarely observed in wild barn owls, it is frequent in captive owls (Bunn *et al*. 1982), and it is also considered to be the major cause of drowning (Shawyer 1987; Barn Owl Trust 2012). Bathing is more likely to reduce OCPs concentrations originated by external contamination associated with airborne particles and dust deposited in feather surface, as these can be more easily washed away with water than preen gland oil (Jaspers *et al*. 2008).

Within the HCH compounds, β-HCH was the dominant isomer in feathers while lindane had the highest concentration of the group in livers. Moreover, lindane concentrations were similar in feathers and livers, suggesting that being a polar compound did not facilitate its deposition during feather formation, as reported in literature (Espín *et al*. 2012; García-Fernández *et al*. 2013). Lindane was also the most common compound detected in river sediments in Portugal, with maximum levels near our study area (Villaverde *et al*. 2008; Carvalho *et al*. 2009). Although we found no comparable data for environmental concentrations of β-HCH, lindane showed greater concentrations than β-HCH in small mammals – the main prey of barn owls – from Central Portugal (Mathias *et al*. 2007). Therefore, the high concentrations of the β-isomer in feathers may have derived from external contamination.

The low concentrations of heptachlor epoxide in Portuguese barn owl feathers and livers compared with the parent compound could result from exposure to recently released heptachlor, and/or from a poor ability of the species to metabolize it. The decreasing trend with age could give some support to the second, if resulting from interferences in the mechanism of heptachlor epoxidation, occurring at hepatic microsomal epoxidases (Gillett and Chan 1968). The mechanism is common to other cyclodiene insecticides (*e.g.* conversion of aldrin and isodrin in their epoxides – dieldrin and endrin, respectively; Gillett and Chan 1968). Therefore, the decrease with age in endrin and dieldrin concentrations, and the concurrent increase in heptachlor could possibly be explained by the interaction between different products and substrates. Other mechanisms could also affect microsomal peroxidases producing a decrease in heptachlor epoxide with age (*e.g.* increase in lipid peroxidation with age; Oropesa *et al*. 2013), therefore the interpretation of its concentrations is difficult in the light of present knowledge.

The interpretation of matrix- and age-related differences in contaminants in low concentrations requires caution, as these may be subjected to concentration-dependent effects. When a contaminant is present in low concentrations (*e.g.* heptachlor epoxide), then feather–liver significant correlations originated by a similar trend may occur irrespectively of the existence of a true relationship in concentrations between matrices. Concentrations of heptachlor in feathers and livers (31% and 11% of ΣOCP, respectively) are much higher than those of heptachlor epoxide (0.2% and 0.3% of ΣOCP). Both contaminants are part of the same metabolic pathway and show similar distributions in feather and liver, but only heptachlor epoxide concentrations showed a significant feather–liver correlation.

5.5.2 Regional variation in organochlorine pesticides concentrations

The high concentrations and ubiquity of HCHs in feathers (mainly β-HCH) in Évora and Tagus is most likely associated with the generalized recent application of lindane in agriculture. Waters draining from farmland soils often contain higher concentrations of this contaminant compared to those draining from forestry soils (Villaverde *et al*. 2008). The presence of lindane in Portuguese sediments has been linked to agricultural areas where historical land use has been rice, wheat or grape crops, and its maximum value (450 ng g^{-1}) has been reported in coastal sediments in an estuary close to our study areas (Villaverde *et al*. 2008). Additionally, concentrations of HCH in terrestrial environment may also be increased by atmospheric transport after volatilization from oceans (Newton *et al*. 2014). Heptachlor is also more abundant in agricultural than forest soils (ATSDR 2007), but since it was banned decades earlier than lindane, the elevated concentrations of the parent contaminant in the barn owl may not result from historical agricultural use. Carvalho *et al*. (2009) reported high concentrations of heptachlor epoxide in sediments of the north bank of the mouth of Sado Estuary, which was conditioned by industrial activity. This estuary is relatively close to our study areas, therefore, we cannot exclude industrial sources as a possible environmental input in our study.

Since there are no significant differences in OCPs concentrations between areas, our results suggest similar temporal use and dosage in Évora and Tagus, despite differences in land uses. Nevertheless, the absence of some OCPs in Tagus which are present in Évora, jointly with a trend for lower OCPs concentrations in Tagus, suggests that this area is in a

more advanced stage of decontamination. This is also supported by the dominance of the parent compound in the ratio DDT:DDE found in estuarine sediments from Sado (partly draining agricultural lands from Évora; 0.21) compared to Tagus (0.18; calculated from Gil and Vale 1999). This may be due to faster degradation of some compounds (such as DDTs) in Tagus, which is favoured by anaerobic conditions such as those encountered in soils that are either periodically or permanently flooded (Wang *et al*. 2007; Hao *et al*. 2008). In opposition, degradation of the ubiquitous HCH isomers is not improved by flooded conditions, and specially β-HCH is apparently not degraded by farming activity (Rubinos *et al*. 2007), which may have contributed to the observed high concentrations.

Among the OCPs detected in Évora but not in Tagus, only ΣDrins concentrations in feathers differed significantly from zero. Differences in individual compounds were probably not significant because only a small number of feather samples were contaminated with some Drins, particularly endrin (22%) and endrin aldehyde (11%). One individual from Évora showed a very high concentration in feather of endrin (1907 ng g^{-1}) and another individual of endrin aldehyde (234 ng g^{-1}). These high values most likely caused ΣDrins to be significantly higher in Évora. Since these individuals were not contaminated with the same compounds in liver, our results suggest episodic current exposure in the area, perhaps resulting from external contamination. This would be possible, for instance, in the presence of obsolete pesticide stocks, kept in such a manner that owls, but eventually not their prey, would have access to endrin and its aldehyde.

5.5.3 Exploring trends in organochlorine pesticides using barn owl feathers and livers

Using as a reference the OCPs concentrations reported by Sierra and Santiago (1987) for barn owl livers from Spain in the 1980s, our results suggest continuous contamination by lindane over the last 30 years, along with an accentuated decrease in heptachlor epoxide, DDT and aldrin. Because lindane was being used as a pesticide at the time of that study, our finding of similar mean concentrations in barn owl liver (36.0 ng g^{-1}; Sierra and Santiago 1987) may be because lindane was one of the latest OCPs banned in Portuguese agriculture, being legally used up to three years before our sampling (Regulation CE 850/2004). β-HCH is a product of degradation of lindane with a great prevalence in agricultural soils, which

accumulates in fat tissue 10–30 times more than the parent compound (Heeschen *et al*. 1980). β-HCH also has been demonstrated as having the lowest degradation ratio of the HCH group (WHO 1992). Therefore, liver concentrations of lindane twice as high those of β-HCH suggest exposure to recently released lindane in Portuguese barn owls. On the other hand, although endosulfan was the most recently banned contaminant in Portugal (two years before sampling; Directive 2006/507/CE), it was the compound detected in lowest concentration and frequency in our study. The faster degradation of endosulfan and its derivates (Kaur *et al*. 1998; Sethunathan *et al*. 2002) is most likely contributing to its low concentrations in barn owls. Endosulfan and lindane were the OCPs most frequently reported above the maximum residue limit in food in Portugal until our sampling (DGPC 2005, 2006; DGADR 2008, 2009b, 2010b, 2011a, 2011b; Fernandes *et al*. 2011; DGAV 2012, 2013, 2014, 2015). However, further conclusions are limited because there is no prior assessment of endosulfan concentrations in barn owl liver, nor information on pesticide use.

Heptachlor epoxide concentrations represent only 0.4% of those reported for the species in Spain in the 1980s (161 ng g^{-1}; Sierra and Santiago 1987), suggesting an accentuated decline. Nevertheless, the great relative percentage of the parent compound in feathers (99%) and livers (97%; Fig. 5.2), and its extremely high concentrations in feathers suggests exposure to recently released heptachlor. Attention should be given to this OCP, which has been excluded from some studies assuming it was no longer emitted in Europe (van der Gon *et al*. 2007), because it may still be present in industrial wastes as, for example, wastewater from coal mining, foundries and nonferrous metals manufacturing (EPA 1995).

In our study DDT was not detected in liver as it was in Spain in the 1980s (50.0 ng g-1; Sierra and Santiago 1987), close to the time of DDT ban in agriculture (Order of 21 May 1976). Half-life of DDT is reported to reach up to 35 years in agricultural soils (Nash and Woolson 1967), therefore it is possible that DDT is available in soil beyond the interval elapsed between its restriction and our sampling. DDT was still allowed in Europe for specific forestry and agricultural uses until 2004, and until 2014 as an intermediate industrial product (Directive 79/17 CEE; Regulation CE 850/2004). Present contamination of Portuguese barn owls with DDT and derivates is most likely due to their great persistence in the environment, since DDE (26% of ΣOCP) was the main compound in terms of relative concentration in liver, comparable with hepatic accumulation of ΣHCH (30% of ΣOCP).

Aldrin was detected in only one individual in concentration representing 3.5% of those reported in Spain (59.0 ng g^{-1}; Sierra and Santiago 1987). In contrast, dieldrin mean concentration in our study doubled that reported 30 years ago (9.00 ng g^{-1}; Sierra and Santiago 1987). These results concur with a decreasing trend of aldrin and a corresponding increasing trend of dieldrin. Dieldrin is much more resistant to biodegradation than aldrin (generally quickly degraded to endrin and dieldrin through epoxidation; Ritter *et al*. 1995), and for that reason aldrin bioaccumulates and biomagnifies mainly in the form of its conversion products (WHO 1989). Therefore, our detection of aldrin in both liver and feathers raises concern, since the compound has been restricted in Portugal ca. 20 years before sampling. Aldrin is still detected in concentrations up to 0.013 µg dm^{-3} in natural springs waters (Cardoso *et al*. 2009), 2.5 ng g^{-1} in estuary sediments (Carvalho *et al*. 2009) and 1.38 µg kg^{-1} in fruits (Fernandes *et al*. 2011), indicating a great persistence of this OCP.

5.6 Conclusions

Our study fills a lack of information on OCPs in Portuguese raptors, showing that the more prominent contaminant groups in barn owl feathers were ΣHCH and ΣHeptachlor, while in livers there was an equitable dominance of ΣHCH, ΣDrins and ΣDDT. Heptachlor, β--HCH, and DDE deserve further attention as we found relatively high concentrations, but aldrin, DDT and derivates also raise some concern due to their persistence.

All the analysed OCPs were detected in the barn owl, suggesting the species may be a good biomonitor of environmental contamination with OCPs. However, interpretations should consider that the high concentrations of β-HCH and heptachlor in feathers may be influenced by external contamination. Feathers may be particularly suitable as biomonitoring tools to detect legacy environmental contaminants which generally occur in residual concentrations (*e.g.* aldrin, DDT), because these may not accumulate in liver while still present in the environment in low concentrations.

6

Conclusions

6 Conclusions

The use of raptors as biomonitors requires that several factors of variation of contamination levels should be considered, so that researchers may discriminate confounding effects and thus identify meaningful patterns of pollutant concentrations. The first part of this thesis focused on confounding sources of Hg variation, mostly linked with access to samples. The second part addressed spatial and temporal patterns in contamination, and examined factors affecting bioaccumulation in the barn owl.

6.1 Sources of variation in sampling procedures

This thesis addressed confounding effects in contamination at two levels: intra--individual and inter-individual variation. Within the individual, we looked at effects of between-feather variation and of time lapse between contamination and feather collection (*i.e.* feather age). At the inter-individual level, we explored the effects of age (*i.e.* variation in accumulation or in exposure to external contamination), spatial variation (*i.e.* variation in exposure) and also revealed OCPs that require further research on their propensity to produce external contamination in feathers.

6.1.1 Intra-individual confounding effects

Feather growth rates, more specifically, the daily increase in mass of a feather, seem to have a negative effect in Hg accumulation. Barn owl feathers show a greater variation in daily increase in mass than in length, and Hg concentrations in primary feathers are better explained by between-feather differences in mass. Therefore, a feather with a slower mass increase will incorporate more Hg per day. Our data with barn owl feathers (Chapter 2) supported that Hg deposition is time-dependent, as suggested by Bortolotti (2010). Nevertheless, the feather-dependent confounding effect is not caused by variation in feather mass, nor has a direct relation with position in the wing, as previously thought. In the barn owl, feather mass and length decrease with inward position in the wing, but Hg concentrations show a different pattern (Chapter 2). Despite there is not a linear relation with position in the wing, further exploitation of our data revealed a different pattern: we could roughly identify the species complex moult pattern in the average Hg concentrations and also in the average excreted Hg amongst primary feathers – starting in P6 and

proceeding sequentially to the inner and outer ends of the wing (Hg is maximum in P6, *i.e.* the first feather to moult, and decreases following the same order). However, a cause-effect relationship between moult pattern and Hg content in these feathers does not exist, since this pattern is observed in first year barn owls (*i.e.* in feathers of the same generation, formed contemporaneously in birds with the same age, without moults). The most plausible explanation is that feather growth rates in the barn owl somehow reflect the species' moult sequence. If this is a characteristic of other bird species, then the growth rate hypothesis is possibly the main explanation to some significant correlations described in the literature between Hg concentrations in feathers and moult sequences (Bortolotti 2010).

Intra-individual differences in feather growth rates, apparently replicating the moult sequence in the barn owl, may also cause a confounding effect related with position in the wing. When analysing relations between Hg concentrations and moult sequences, the interpretation may also be influenced by variations with feather age (intra-individual variation), in addition to the effect of the individuals' age (see 6.1.2 Inter-individual confounding effects). The barn owl has protracted feather replacement (Baker 1993, Martínez *et al*. 2012, Demongin 2016). Consider a barn owl on its second year, which had already moulted P6: this feather is expected to contain a high Hg concentration, both because it has grown slower and because it may reflect enhanced bioaccumulation compared to the other unshed primary feathers. However, when the same owl is on its fourth year, it shed P6 two years before, P7 and P8 one year before and may have recently shed P9. Since Hg concentrations decrease outwards from P6 (possibly owing to increasing feather growth rates), it is possible that the raise in the same direction in Hg concentration following moult (*i.e.* caused by a yearly accumulation in the Hg body burden that may result from a yearly raise in the Hg available in blood to deposition in feathers) is such that concentrations between primaries become more even. Thus, it is possible that the moult is actually masking differences among feathers rather than accentuating them. This could be the reason why Dauwe *et al*. (2003) found no relation between Hg concentrations in barn owl feathers and the moult sequence.

Feather age represents the time lapse between contaminant incorporation and sampling. For that reason, it may have a confounding effect in comparisons with the internal levels of the individual. Contrarily to internal tissues, concentrations in feathers remain

stable from the moment feather reaches its full length and circulation is interrupted (Furness *et al*. 1986). Therefore, the timing of sampling may not correspond to the timing of contaminant uptake in feathers, what may increase differences with internal organs/tissues. This should be the explanation why only one in sixteen OCPs analyzed in this thesis (heptachlor epoxide) showed a positive significant correlation between feathers and livers (Chapter 5). Nevertheless, the interpretation of this correlation is complex and our results suggest a possible concentration-dependent effect: (1) concentrations of heptachlor in feathers and livers are much higher than those of heptachlor epoxide, (2) both contaminants are part of the same metabolic pathway – heptachlor being degraded in the more stable heptachlor epoxide – and show similar distributions in feather and liver, and (3) only heptachlor epoxide concentrations showed a significant feather-liver correlation. Feather-liver significant correlations may therefore have been originated by a similar negative trend with age, resulting in similarities between concentrations in the two matrices with independent causes.

All considered, confounding effects in intra-individual variation in Hg and OCPs concentrations should be taken into account when interpreting correlations between feather concentrations and matrix-related variables. This thesis provided evidence that redundant variation may create similar trends that may be deprived of a biological meaning, and in such cases may mislead conclusions in between-feather and between-matrix comparisons.

6.1.2 Inter-individual confounding effects

The time lapse between contamination and sampling in one individual, measured as feather age, may also increase inter-individual variation if feathers with different ages are used to compare different individuals. Collection of barn owl feathers in opportunistic sampling is age-biased towards un-moulted feathers, because there is an easier access to nestlings and first-year road-killed individuals. Moreover, individuals in the second calendar year can still show no moults and older individuals may still conserve several un-moulted feathers. Thus, many of the collected barn owl feathers do not reflect the age of the individual and the collection of an adequate amount of recently grown feathers may be challenging (Chapter 3). For this reason, research on age-related variation in the barn owl

should not rely on opportunistic sampling. Nonetheless, this thesis provided evidence that opportunistic sampling of barn owl feathers most likely contributes to minimize the confounding effect of redundant age-related variation. Moreover, when several feathers per territory (mixing nestlings and adults) were used to calculate the mean Hg concentration, age-effects resulted to be negligible (Chapter 3).

Beyond differences in bioaccumulation, age may also represent spatial variation, *i.e.* displacement from sampling site and contamination site, which depends on the life-history of the individuals. Road-kill mortality is higher in the barn owl during post-fledging dispersal, and carcasses are often collected several kilometres away from their places of birth (progenitors' territories). In a radio-tracking study carried out on our study area, 5 of 13 owls that died during dispersal were collected 8 to 60 km away from their natal site (I. Roque, A. Marques, T. Marques, R. Lourenço, J. E. Rabaça, unpublished data). Despite the chance of locating a dispersing owl near its birth place should be much higher than far from it, this study gives evidence that a feather collected from road-killed first year barn owls has a probability of at least 38% of not representing Hg contamination at the collection site. In an adult bird, this probability should be much higher: from a total of 120 broods, monitored during 9 years in a total of 52 nests, none of the 376 owls ringed as nestlings was found breeding at the original territory (I. Roque, A. Marques, R. Lourenço, J. E. Rabaça, unpublished data). Despite breeding adults are expected to be collected near their territories, feathers from the first moult do not represent contamination in collection site. Nevertheless, this thesis provided evidence that when several feathers per territory and per road-killed individual are used to calculate a mean Hg concentration, results are comparable among age classes in feathers collected in nests and in road-killed owls in a same region. So, road-killed barn owls apparently provide useful information to characterize Hg contamination at a regional scale, despite their disconnection to specific contamination sites, thus suggesting that neither age nor spatial variation produce relevant confounding effects at the regional level (Chapter 3), provided that: (1) sample size is relatively large, (2) landscape and land uses are similar, and (3) the limit should not extended beyond 60 km apart from the sampling sites of the road-killed barn owls, however this distance may vary in other regions.

Another factor that may increase inter-individual redundant variation is external contamination. Despite this confounding effect is in general negligible in Hg (Burger and Gochfeld 1997; Dauwe *et al*. 2003), there is not a total agreement on its relevance in OCPs, given the relative novelty of the use of feathers for monitoring these contaminants (Espín *et al*. 2016). Further research is needed on the susceptibility of several OCPs to producing external contamination in feathers. In this regard, this thesis prompted interest in a few OCPs: β-HCH, heptachlor, endrin and endrin aldehyde. The high concentrations of β-HCH could result from external contamination, because this OCP is apparently less abundant than lindane (γ-HCH, which can be its parent compound) in the environment and in food (Villaverde *et al*. 2008; Mathias *et al*. 2007). For heptachlor, there is no equivalent information, and an alternative (or possibly cumulative) explanation for the high concentrations in feathers could be a poor ability of the barn owl to metabolize this OCP (*e.g.* interferences of different products and substrates in the mechanism of heptachlor epoxidation, occurring at hepatic microsomal epoxidases; Gillett and Chan 1968). On the other hand, endrin and endrin aldehyde were detected in high concentrations in feathers (each OCP in only one individual), while not concurrently present in livers of the same individuals. These findings were restricted to a particular region (Évora), suggesting occasional exposure. One possible explanation could be the existence of obsolete pesticide stocks, providing access of owls (but apparently not their prey) to endrin and its aldehyde (Chapter 5).

6.1.3 Recommendations to sampling procedures

A sound knowledge of the species biology and ecology, in order to understand how these can influence intra- and inter-individual variability, is crucial in biomonitoring contaminants with raptors. This thesis provides evidence that different feather types (body and flight feathers) may be interchangeably collected to estimate Hg concentrations in un--moulted barn owls, regardless of feather size and position in the wing for remiges. The best criterion for minimizing redundant variation is calculating an average concentration from several feathers from the same individual. Under a restrictive sampling scenario in which only one flight feather could be analysed, this should be selected among the five innermost primaries (P1–P5). Based on mean feather mass (because feather mass affects Hg concentrations), this group showed the lowest average deviations from sample and

individual mean Hg concentrations. In case the position of remiges cannot be identified, this group roughly corresponds to the range of length of 157–194 cm.

If several feathers are combined to calculate a mean value for the brood or for the territory, calculating an average concentration from several feathers from different individuals also results in the attenuation of age-related redundant differences in Hg concentrations. Feather samples collected from road-killed barn owls and in nest sites may be mixed to characterize background Hg contamination in a broader area because samples from road-killed owls are apparently representative of Hg contamination in territories, possibly in the range of 60 km from collection sites. Opportunistic sampling of barn owl feathers apparently contributes to reduce redundant variation of age-related confounding effects in Hg concentrations, because it is biased towards un-moulted feathers. For this reason research or applied studies focused on age-related variation in the barn owl should require directed sampling. In this regard, we suggest the collection of body feathers from nestlings and also the capture of adults in or near nests for collection of body feathers, which should be moulted every year, usually between July and September (Bunn *et al*. 1982). Plucking recently grown flight feathers (from the tail or wing, be it a primary, secondary or rectrix feather) should be avoided, since in raptors those feathers may not be readily replaced nor grow normally in the next moult (Delnatte 2014). Moreover, plucking these feathers apparently has significant post-sampling costs on flight performance (McDonald and Griffith 2011).

Most likely the criterion of calculating average concentrations from several feathers is also applicable to other contaminants besides Hg, including OCPs. Nevertheless, the sources of variation in sampling procedures in OCPs were not in the scope of this thesis, and despite the effort to examine patterns in OCPs contamination, we have not established a basis for recommendations to sampling procedures.

6.2 The barn owl as biomonitor of environmental contamination

This thesis also addressed the suitability of the barn owl as a biomonitor of environmental contamination, evaluating variation patterns in Hg and OCPs with regard to: (1) identifying contaminants of concern (Hg and OCPs) and (2) inspecting factors affecting variation in bioaccumulation (Hg).

6.2.1 Contaminants of concern in barn owls

All the contaminants evaluated in this thesis were detected in barn owl feathers, and therefore this matrix seems to be a good biomonitoring tool for Hg and OCPs. Heptachlor, β--HCH, and DDE (the latter revealed by liver concentrations) deserve further attention as we found relatively high concentrations, but DDT (detected only in feathers) and aldrin/dieldrin also raise some concern due to their persistence. We cannot exclude the possibility of external contamination from the two contaminants that produced the most accentuated contamination in feathers (β-HCH and heptachlor), meaning it is not clear if these may result in adverse effects. However, this information is still relevant in terms of environmental contamination, showing that: (1) the most resistant isomer of the HCH family of compounds (β-HCH) is present in extremely high concentrations in south Portugal; and (2) heptachlor, a contaminant that has not received much attention, because it was banned in Europe, is likely to have a current impact in Portugal. Lindane was among the latest contaminants banned in Portuguese agriculture; consequently, its concentrations in barn owls most likely result from recent exposure. In contrast, the DDT family of compounds raises concern for barn owls for its persistence. DDE showed the major relative concentrations in liver, similar to those of total HCH. This means that legacy compounds, usually present in trace concentrations in the environment, may represent a burden similar to that of recently banned OCPs in barn owl internal tissues. The great propensity of legacy OCPs for bioaccumulation is worrying, because the extent of adverse effects caused by sub-lethal contamination remains unknown, and these compounds are potentially more toxic than more recently used pesticides. Additionally, the detection of aldrin and related compounds in both feathers and livers might also be of concern. Because dieldrin (the product of aldrin epoxidation) was associated with a greater impact on raptor mortality (resulting from high concentrations; Table 6.1) than on reproduction (Newton *et al*. 1991), the current trace environmental concentrations of DDT--related compounds are probably more harmful for barn owls than those of aldrin-like OCPs. Nevertheless, dieldrin might have been increasing in Iberian barn owls in the last three decades (Sierra and Santiago 1987; Chapter 5), what was not expected because these cyclodien insecticides were banned in Portugal ca. 20 years before sampling (Ordinance 660/88). Despite the maximum hepatic concentrations of some contaminants of concern in barn owls are currently much lower than the lower limit of lethal range for the species (Table

6.1), the range of concentrations that may impair reproduction in barn owls is still unknown. Impaired reproduction in barn owls, which may be causing significant eggshell thinning, egg breakage, embryo mortality, and reduced production per pair, has been associated with legacy contaminants like DDE (Mendenhall *et al*. 1983). Moreover, considering the negative trend that was recently revealed for the Portuguese barn owl population (Lourenço *et al*. 2015) and the evidence provided by this thesis on the accumulation in liver of considerable concentrations of some legacy OCPs (mainly DDE), further investigation is needed with regarding the effect assessment of legacy OCPs in the species, and in particular in the Portuguese barn owls.

Mercury concentrations in barn owls were lower than those previously reported for the species in Portugal (1.20 mg kg^{-1}; Chapter 2) and for Portuguese raptors (1.25 mg kg^{-1}; Chapter 1). Nevertheless, effect assessment is lacking and therefore the potential toxicity of the measured concentrations for the barn owl is still unknown. Since Hg is probably the metal of most concern for adverse biological effects (Walker *et al*. 2001), its increasing trend in biota (Froslie *et al*. 1986) reinforces the need for assessment of effects at sub-lethal levels. In our data set, five samples from two individuals showed Hg concentrations in the range of the values reported to produce negative effects on terrestrial birds (2.8–4.9 mg kg^{-1}). Therefore, despite in our study area barn owls are in general not exposed to very high Hg contamination, we should consider some of our values as sufficiently high to potentially impair reproduction.

Table 6.1 Maximum hepatic concentrations in Portuguese barn owls (this thesis) and lethal range of hepatic concentrations for the species (Newton *et al*. 1991)

Contaminant	**Maximum hepatic concentrations ($ng\ g^{-1}$) in Portuguese barn owls**	**Lethal range of hepatic concentrations (ppm)**
Heptachlor epoxide	2.98 ng g^{-1}	14.4–26.0 ppm
	Máx. ΣHeptachlor: 7 940 ng g^{-1}	(*i.e.* from 14 400 ng g^{-1})
HEOD	Aldrin: 198 ng g^{-1}	6.00–44.0 ppm
(aldrin and dieldrin)	Dieldrin: 93.9 ng g^{-1}	(*i.e.* from 6 000 ng g^{-1})
	Máx. ΣDrins: 2 001 ng g^{-1}	
DDE	162 ng g^{-1}	130–270 ppm
	Máx. ΣDDT: 247 ng g^{-1}	(*i.e.* from 130 000 ng g^{-1})

6.2.2 Factors affecting bioaccumulation in the barn owl

This thesis provided evidence that biota-soil accumulation factors (BSAFs) in raptors may be helpful tools for understanding contaminant dynamics in terrestrial systems, allowing for detaching diet-related variation in bioaccumulation while visualizing spatial patterns. Biota-soil accumulation factors simulate a one-compartment model, excluding the effect of biomagnification in bioaccumulation, which is known to be of major importance in top predators. For this reason, BSAFs have never been used in terrestrial ecotoxicological studies for other organisms than invertebrates, fungi and plants (Chapter 4). However, we address that isolating the variability inherent to the habitat facilitates general patterns that may be useful to raise hypothesis regarding habitat effects on bioaccumulation, through possible effects on diet-related variation in raptors.

Bioaccumulation in the barn owl seems to be affected by agricultural land uses, which interfere with both concentrations in soil and in feathers, but also on Hg transfer pathways along the food web. This thesis provided evidence that permanently irrigated and patchy agricultural areas may possibly be used as proxy of lower Hg agricultural input and/or faster Hg decontamination. These, and also areas with permanent crops (which were associated with lower Hg contamination in feathers) should be further inspected for variations in food webs, because of potential variations in prey Hg bioaccumulation, diversity, abundance and/or accessibility (Chapter 4). Habitat may therefore be a mediator of bioaccumulation along the food web, because it determines plant composition and distribution, and prey availability, which in turn may affect Hg distribution in soil, and induce variability in the way the contaminant is transferred to the barn owl. In other words, habitat may possibly be a surrogate of the pathways leading Hg from the soil to the barn owl.

Spatial patterns in other contaminants also seem to support the possible influence of land uses in exposure. Lower concentrations of parent compounds in relation to their degradation products (DDT:DDE) were previously reported in sediments from the Tagus estuary, draining agricultural soils from our study area where less contaminants were detected in barn owl feathers (Gil and Vale 1999; Chapter 5). We hypothesized that this possibly corresponds to a decontamination effect, because faster degradation of some compounds (such as DDTs) is favoured by anaerobic conditions in soils that are periodically

or permanently flooded (Wang *et al*. 2007; Hao *et al*. 2008). Permanently irrigated agricultural areas are more abundant in the Tagus area than in Évora. Additionally, irrigation may increase elimination of elemental Hg (Gustin and Stamenkovic 2005; Chapter 4). Since inorganic Hg is methylated, concentrated, and transported in the direction of water flow in wetlands (Ackerman *et al*. 2010; Ackerman and Eagles-Smith 2010), this may correspond to an increased probability of off-site contamination. In opposition, degradation of the ubiquitous HCH isomers, is not improved by flooded conditions, and specially β-HCH is apparently not degraded by farming activity (Rubinos *et al*. 2007), which may have contributed to high concentrations in barn owl feathers (see 6.2.1 Contaminants of concern in barn owls).

Landscape diversity, here characterized by small-scale land use heterogeneity, seems to affect bioaccumulation, mainly by influencing small-scale Hg distribution. We hypothesised that small-scale soil conditions and vegetation patterns may influence Hg retention in soil, mimicking the wider-scale effect of plant productivity driven by water availability (Obrist *et al*. 2016; Chapter 4). More specifically, a small-scale process dependent on irrigation (as for rainfall deposition) and soil physical-chemical properties (as for geomorphic variation) may create small-scale patterns in soil Hg concentrations, with consequences in Hg bioaccumulation (Eagles-Smith *et al*. 2016; Obrist *et al*. 2016).

Complementary, greater landscape diversity may be associated with more diverse plant and animal communities, which may create a larger number of pathways for Hg to reach top predators, such as the barn owl. Nevertheless, interpreting how the resulting heterogeneous Hg distribution could influence bioaccumulation is complex, requiring further studies with larger sample size (*i.e.* multivariate models) in order to explore the effect of landscape diversity in Hg and its relations with food web structure.

6.2.3 Is the barn owl a good biomonitor species?

The barn owl adequately meets the requirements of a biomonitor species, according with Becker (2003), by contributing to: (1) little risk of monitoring being confounded by uncertainties or misinterpretations (*i.e.* is easy to identify and has a well known biology and ecology); (2) representing contamination by biomagnification (*i.e.* is at top positions in the food webs); (3) relatively easy access to non-destructive or minimally-invasive sampling

procedures (*i.e.* blood, feathers); (4) distinctly representing changes in the environment (*i.e.* has a low reproductive output); (5) allowing comparisons between different ecosystems, countries and continents (*i.e.* has a widespread distribution). Moreover, the barn owl is a charismatic species, capable of raising public interest, therefore being a good candidate to becoming a sentinel species. Its diet is also very well studied, because of access to pellets containing a larger number of undigested prey remains than in other owl species, potentially allowing for the application of correction factors to variation in biomagnification. Based on its diet, the barn owl has been considered one of the most appropriate raptor species for monitoring the risk of secondary poisoning of strongly bioaccumulative contaminants, including DDT, dieldrin, lindane and MeHg (Jongbloed *et al*. 1994). This thesis added several advantages for using the barn owl with respect to access to samples and to control redundant variation (*e.g.* feather type and age in Hg concentrations) in opportunistic sampling procedures, and also to potentially correct possible habitat-related effects in bioaccumulation.

Similarly to barn owls, other raptor species should be good biomonitors, which could be used worldwide to study local contamination, and above all, the potential effects of contaminants on the environment. However, as highlighted throughout this thesis, using raptors as biomonitors requires that researchers have a good background information and knowledge on several factors (Table 6.2). This comprehensive approach will allow stronger and breakthrough conclusions based on contaminant concentrations in raptors.

Table 6.2 Factors to take into account when using raptors as biomonitor species

Factor	Rationale
Raptor species	The biology, ecology, behaviour, and abundance of each species may have strong implications on accessibility to samples (feathers, blood, etc.), spatial distribution, migratory behaviour, etc.
Diet	Intra-specific diet variations influence biomagnification, having implications on the measured differences in contamination among trophic levels and in the environment (soil, water)

Table 6.2 Factors to take into account when using raptors as biomonitor species (continued)

Factor	Rationale
Age and sex	Individuals of different ages and sexes may differ in concentration due to variations in diet and movement ecology (home range size, dispersal, migration)
Matrix	Contaminant concentrations in feathers (as excretory pathways) and internal tissues, (with different bioaccumulation and biotransformation capability) are subjected to variations that should be inspected for meaningful relationships with effects
Feather type	In an analysis based in a single feather, intra- and inter-individual variation should be minimized because there are factors affecting contaminant concentrations that may result in differences between feather types (*e.g.* mass dilution, probably caused by variation in growth rates, in Hg concentrations)
Contaminants	Depending on which compound the study is directed to, all the above information should be considered to maximise results

Using raptors as biomonitors may also have some limitations, depending on the biomonitoring aims: (1) raptors may not reflect site-specific contamination (given their mobility, which may result in displacement of sampling and contamination sites); (2) it may be difficult to isolate specific factors affecting contamination (given the large number of factors regulating their populations); and (3) collection of invasive samples and laboratory studies are difficult (because of ethic, legal and conservation constraints). The advantages and limitations of using raptors in a monitoring study should be clearly identified and assessed in advance, so that sampling design is planned to reduce or eliminate the possible disadvantages (Becker 2003).

6.3 Recommendations to further research

Feathers have been used in exposure assessment for several contaminants. However, in order to leverage the potential use of these biomonitoring tools, the interpretation of concentrations needs further research on (1) understanding the effect of feather growth rates on contaminant deposition, (2) discerning external contamination and (2) linking exposure to effects. The latter involves a complex and resource demanding integrated analysis (Figure 6.1). Therefore, the selection of species and contaminants should minimize

redundant variation, maximize access to samples and benefit from the existing body of knowledge. We advocate that the barn owl and the compounds evaluated in this thesis are good candidates. For major environmental contaminants like dieldrin, DDE and Hg, minimum critical levels for raptors reproductive effect in eggs, and for acute effects on internal organs are available (Noble and Elliot 1990; Peakall *et al*. 1990; Newton *et al*. 1992). The assessment of minimum critical concentrations in feathers should include measurement of concentrations in blood at time of feather formation and corresponding levels in target organs (which depend on the contaminant being analyzed). This would only be possible during feather formation in nestlings, or during moult in adult birds. Then, it should be examined if concentrations in feathers could possibly be used as proxy of concentrations in target organs for specific contaminants in effect assessments. If this is feasible, the use of nestlings as a standard would allow for excluding age-related confounding effects. Moreover, since the access to samples would involve nest monitoring, breeding parameters could be collected simultaneously, facilitating effect assessment in the population. In order to access effects in individuals, the blood collected for examine feather-internal tissue relations could also be used for identifying biomarkers. For instance, DDE concentrations of 16 ppm in barn owl eggs were associated with nest failure (Klaas *et al*. 1978): could a correspondence be made with feather concentrations? Several blood clinical-chemical parameters were correlated with DDE concentrations in raptors, including albumin and total protein, which reflect health and homeostasis of liver (Sonne *et al*. 2012), a target organ for DDT-like OCPs (Jongbloed *et al*. 1994; Chapter 5): could the inclusion of such biomarkers reveal potential health effects, that could also be related with feather concentrations?

Additionally, factors affecting bioaccumulation should be inspected, in order to access if there is a need for applying correction factors to feather concentrations. Correction factors are normally used to apply laboratory-based concentrations to field estimation, in order to improve reliability of risk assessment. The high variation in contaminant concentrations in the soil within territories of raptors also hampers risk assessment (Traas *et al*. 1996). Therefore, diet, land use and landscape diversity are good candidates to correction factors. However, the relevant source of variation that requires correction should be identified in advance. For instance, differences within and between species in sensitivity to

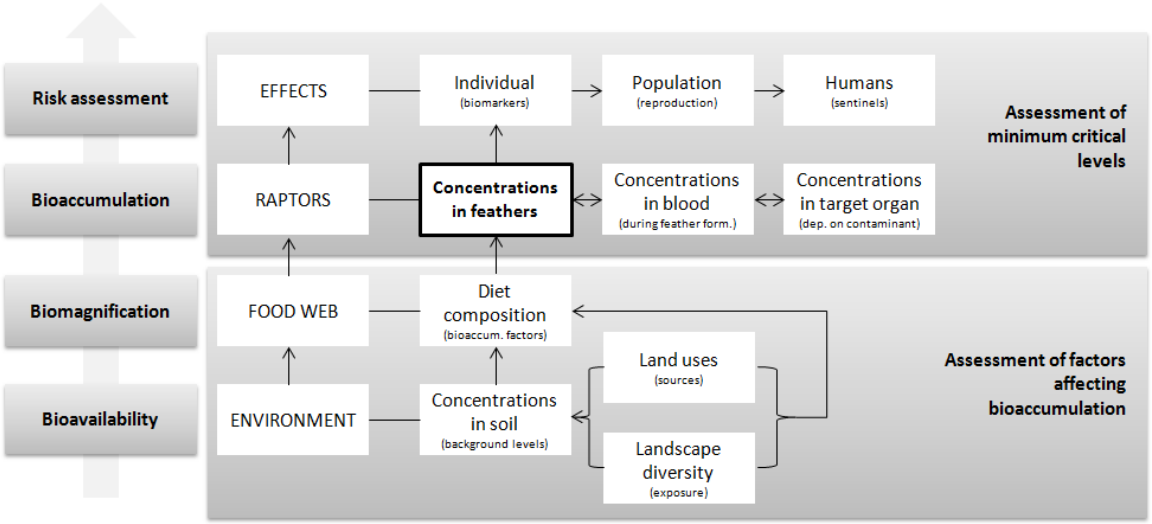

Figure 6.1 Model proposed for an integrated analysis of the relationship between exposure and effects of environmental persistent contaminants, based on raptor feather concentrations

lipophilic contaminants may be explained by differences in total fat content. For that reason, the previously suggested correction for caloric content, assimilation of food types and metabolic rates may be of minor importance in assessing species sensitiveness (*i.e.* no--observed-effect concentrations). Sensitiveness of raptors to DDT can be used, for instance, for derivation of soil quality criteria (*i.e.* maximum permissible concentration) based on bioaccumulation of soil contaminants in terrestrial food webs (Traas *et al*. 1996). Considering that the barn owl may have considerable variation in diet, depending on food supply, which in turn depends on the habitat (de Bruijn 1994; Chapter 4), further investigation on these sources of variation and their inter-relation should provide a good basis for assessing the need of correction factors for feather concentrations.

Given the considerable complexity of the ecological models, which still cannot accurately represent reality, the assessment of entangled patterns may be facilitated by partitioning the variation, in order to observe simpler relationships. Compartmenting variation in ecotoxicological studies may raise new hypotheses, facilitating the assessment of the relevance of specific issues for representing the whole system. Likewise, in the use of feathers as biomonitoring tools, it is important to isolate factors that contribute to redundant variation, in order to identify patterns with biological meaning.

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